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(54) DNA sequences useful for the synthesis of carotenoids.

(57) Disclosed are DNA sequences which are useful for the synthesis of carotenoids such as lycopene, β-carotene, zeaxanthin or zeaxanthin-diglucoside, that is, DNA sequences encoding carotenoid biosynthesis enzymes. These DNA sequences are the sequences ① - ⑥, respectively, shown in the specification.

Also disclosed is a process for producing a carotenoid compound which is selected from the group consisting of prephytoene pyrophosphate, phytoene, lycopene, β-carotene, zeaxanthin and zeaxanthin-diglucoside, which comprises transforming a host with at least one of the DNA sequences ① - ⑥ described above and culturing the transformant.

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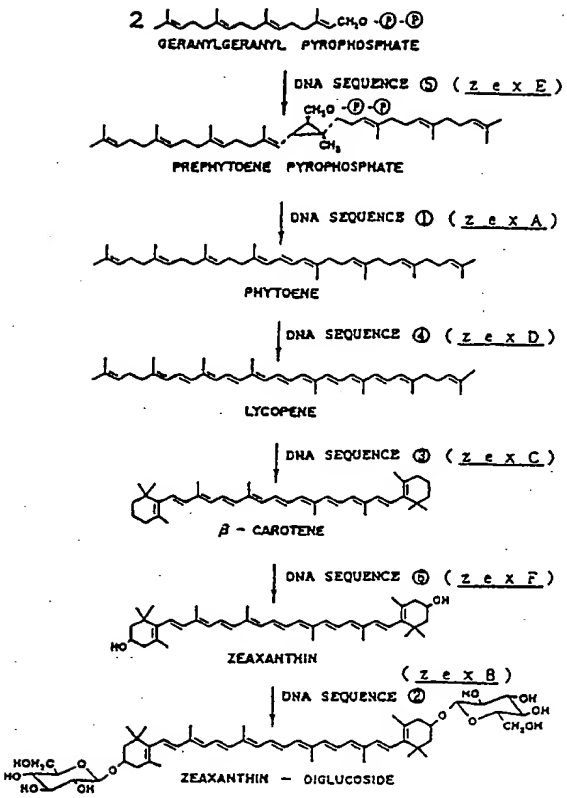

2
GERANYLGERANYL PYROPHOSPHATE
DNA SEQUENCE ⑤ (z e x E)
PREPHYTOENE PYROPHOSPHATE
DNA SEQUENCE ① (z e x A)
PHYTOENE
DNA SEQUENCE ④ (z e x D)
LYCOPENE
DNA SEQUENCE ③ (z e x C)
β - CAROTENE
DNA SEQUENCE ⑥ (z e x F)
ZEAXANTHIN
(z e x B)
DNA SEQUENCE ②
ZEAXANTHIN - DIGLUCOSIDE

FIG. 8

DNA SEQUENCES USEFUL FOR THE SYNTHESIS OF CAROTENOIDS

BACKGROUND OF THE INVENTION

Field of the Art

The present invention relates to DNA sequences which are useful for the synthesis of carotenoids such as lycopene, β-carotene, zeaxanthin or zeaxanthin-diglucoside.

The present invention also relates to processes for producing such carotenoid compounds.

Related Art

Carotenoids are distributed widely in green plants. They are yellow-orange-red lipids which are also present in some mold, yeast and so forth, and have recently received increased attention as natural coloring materials for foods. Among these carotenoids, β-carotene is a typical one, which is used as a coloring materials and as a precursor of vitamin A in mammals as well. It is also examined for its use as a component for preventing cancer [see, for example, SHOKUHIN TO KAIHATSU (Foods and Development), 24, 61-65 (1989)]. Carotenoids such as β-carotene are widely distributed in green plants, so that the plant tissue culture has been examined for the development of a method for producing carotenoids in a large amount which is free from the influence of natural environment [see, for example, Plant Cell Physiol., 12, 525-531 (1971)]. The examination has been also made for detecting a microorganism such as mold, yeast or green algae which is originally high carotenoid productive and for producing carotenoids in a large amount with use of such microorganism (see, for example, The Abstract of Reports in the Annual Meeting of NIPPON HAKKO KOGAKU-KAI of 1988, page 139). However, neither of these methods are successful at present in producing β-carotene at a good productivity which exceeds the synthetic method in commercial production of β-carotene. It would be very useful to obtain a gene group which participates in the biosynthesis of carotenoids, because it will be possible to produce carotenoids in a large amount by introducing a gene group which has been reconstructed to express proper genes in the gene group in a large amount, into an appropriate host such as a plant tissue culture cell, a mold, an yeast or the like which originally produces carotenoids. Such a development in technology has possibilities for finding a method of producing β-carotene superior to the synthetic method and a method of producing useful carotenoids other than β-carotene in a large amount.

Furthermore, the synthesis of carotenoids in a cell or an organ which produces no carotenoid will be possible by obtaining the gene group participating in the biosynthesis of carotenoids, which will add new values to organisms. For example, several reports have recently been made with reference to creating flower colors which cannot be found in nature by using genetic manipulation in flowering plants [see, for example, Nature, 330, 677-678 (1987)]. The color of flowers is developed by pigments such as anthocyanine or carotenoids. Anthocyanine is responsible for flower colors in the spectrum of red-violet-blue, and carotenoids are responsible for flower colors in the spectrum of yellow-orange-red. The gene of the enzyme for synthesizing anthocyanine has been elucidated, and the aforementioned reports for creating a new flower color are those referring to anthocyanine. On the other hand, there are many flowering plants having no bright yellow flower due to no function of synthesizing carotenoids in petal (e.g. petunia, saintpaulia (african violet), cyclamen, Primula malacoides, etc.). If suitable genes having been reconstructed so as to be expressed in petal in a gene group referring to the biosynthesis of carotenoids are introduced into these flowering plants, the flowering plants having yellow flowers will be created successfully.

However, enzymes for synthesizing carotenoids or genes coding for them have been scarcely elucidated at present. The nucleotide sequence of the gene group participating in the biosynthesis of a kind of carotenoids has been elucidated lately only in a photosynthetic bacterium Rhodobacter capsulatus [Mol. Gen. Genet., 216, 254-268 (1989)]. But this bacterium synthesizes the acyclic xanthophyll spheroidene via neurosporene without cyclization and thus cannot synthesize general carotenoids such as lycopene, β-carotene and zeaxanthin.

There are prior arts with reference to yellow pigments or carotenoids of Erwinia species disclosed in J. Bacteriol., 168, 607-612 (1986), J. Bacteriol., 170, 4675-4680 (1988) and J. Gen. Microbiol., 130, 1623-1631 (1984). The first one of these references discloses the cloning of a gene cluster coding for yellow pigment

synthesis from Erwinia herbicola Eho 10 ATCC 39368 as a 12.4 kilobase pair (kb) fragment. In this connection, there is no illustration of the nucleotide sequence of the 12.4 kb fragment. The second literature discloses the yellow pigment synthesized by the cloned gene cluster, which is indicated to belong to carotenoids by the analysis of its UV-visible spectrum. The last literature indicates that the gene participating in the production of a yellow pigment is present in a 260 kb large plasmid contained in Erwinia uredovora 20D3 ATTC 19321 from the observation that the yellow pigment is not produced on curing the large plasmid, and further discloses that the pigment belongs to carotenoids from the analysis of its UV-visible spectrum.

However, the chemical structures of carotenoids produced by the Erwinia species or of its metabolic intermediates, enzymes participating in the synthesis of them or the nucleotide sequence of the genes encoding these enzymes remain unknown at present.

DISCLOSURE OF THE INVENTION

Outline of the Invention

The object of the present invention is to provide DNA sequences which are useful for the synthesis of carotenoids such as lycopene, β-carotene, zeaxanthin or zeaxanthin-diglucoside, that is DNA sequences encoding carotenoid biosynthesis enzymes.

In other words, the DNA sequences useful for the synthesis of carotenoids according to the present invention are the DNA sequences ① - ⑥ described in the following (1) - (6).

(1) a DNA sequence encoding a polypeptide which has an enzymatic activity for converting prephytoene pyrophosphate into phytoene and whose amino acid sequence corresponds substantially to the amino acid sequence from A to B shown in Figs. 1-(a) and (b) (DNA sequence ①);

(2) a DNA sequence encoding a polypeptide which has an enzymatic activity for converting zeaxanthin into zeaxanthin-diglucoside and whose amino acid sequence corresponds substantially to the amino acid sequence from C to D shown in Figs. 2-(a) and (b) (DNA sequence ②);

(3) a DNA sequence encoding a polypeptide which has an enzymatic activity for converting lycopene into β-carotene and whose amino acid sequence corresponds substantially to the amino acid sequence from E to F shown in Figs. 3-(a) and (b) (DNA sequence ③);

(4) a DNA sequence encoding a polypeptide which has an enzymatic activity for converting phytoene into lycopene and whose amino acid sequence corresponds substantially to the amino acid sequence from G to H shown in Figs. 4-(a), (b) and (c) (DNA sequence ④);

(5) a DNA sequence encoding a polypeptide which has an enzymatic activity for converting geranylgeranyl pyrophosphate into prephytoene pyrophosphate and whose amino acid sequence corresponds substantially to the amino acid sequence from I to J shown in Figs. 5-(a) and (b) (DNA sequence ⑤); and

(6) a DNA sequence encoding a polypeptide which has an enzymatic activity for converting β-carotene into zeaxanthin and whose amino acid sequence corresponds substantially to the amino acid sequence from K to L shown in Fig. 6 (DNA sequence ①).

Another object of the present invention is to provide processes for producing carotenoid compounds.

More specifically, the present invention also provides a process for producing a carotenoid compound which is related from the group consisting of prephytoene pyrophosphate, phytoene, lycopene, β-carotene, zeaxanthin and zeaxanthin-diglucoside, which comprises transforming a host with at least one of DNA sequences ① - ⑥ described above and culturing the transformant.

Effect of the Invention

The successful acquirement of the gene group (gene group encoding the biosynthetic enzymes of carotenoids) useful for the synthesis of carotenoids such as lycopene, β-carotene, zeaxanthin, zeaxanthin-diglucoside or the like according to the present invention has made it possible to produce useful carotenoids in large amounts, for example, by creating a plasmid in which the gene(s) can be expressed in a large amount and employing an appropriate plant tissue culture cell, a microorganism or the like transformed with the plasmid. The success in acquiring the gene group useful for the synthesis of

carotenoids such as lycopene, β-carotene, zeaxanthin, zeaxanthin-diglucoside or the like according to the present invention has made it possible to synthesize carotenoids in cells or organs which produce no carotenoid by creating a plasmid in which the gene(s) can be expressed in a target cell or organ and transforming a suitable host with this plasmid.

DETAILED DESCRIPTION OF THE INVENTION

The DNA sequences according to the present invention are the aforementioned DNA sequences ① - ⑥, that is, genes encoding the polypeptides of respective enzymes which participate in the biosynthesis reaction of carotenoids, in particular, for example, such polypeptides in Erwinia uredovora 20D3 ATCC 19321.

A variety of gene groups containing the DNA sequences of a combination of a plurality of sequences among these DNA sequences ① - ⑥ can be expressed in a microorganism, a plant or the like to afford them the biosynthesis ability of carotenoids such as lycopene, β-carotene, zeaxanthin, zeaxanthin-diglucoside or the like. The respective DNA sequences constructing the gene group may be present on a DNA strand or on different DNA strands individually, or optionally, the respective DNA sequences may comprise a plurality of DNA sequences present on a DNA strand and a DNA sequence present on another DNA strand.

The aforementioned gene group encode the polypeptides of a plurality of enzymes participating in the production of carotenoids. A recombinant DNA is created by incorporating the gene group into a proper vector and then introduced into a suitable host to create a transformant, which is cultured to produce mainly in the transformant a plurality of enzymes participating in the formation reaction of carotenoids and to conduct the biosynthesis of carotenoids in the transformant by these enzymes.

The DNA sequence shown in Fig. 7-(a) to (g), which is an example according to the present invention, is acquired from Erwinia uredovora 20D3 ATCC 19321 and thus exhibits, as illustrated in the experimental example below, no homology in the DNA-DNA hybridization with the DNA strand containing the gene group for synthesizing the yellow pigment of Erwinia herbicola Eho 10 ATCC 39368 (see Related Art described above).

DNA Sequences encoding the polypeptide of each enzyme

The DNA sequences of the present invention are the DNA sequences ① - ⑥ (or the DNA strands ① - ⑥), respectively. Each of the DNA sequences contains a nucleotide sequence encoding the polypeptide whose amino acid sequence corresponds substantially to such an amino acid sequence as in the aforementioned specific regions in Figs. 1 - 6 (for example, from A to B in Fig. 1). In this connection the term "DNA sequence" means a polydeoxyribonucleic acid sequence having a length. In the present invention, the "DNA sequence" is defined by an amino acid sequence of a polypeptide which is encoded by the DNA sequence and has a definite length as described above, so that each DNA sequence has also a definite length. However, the DNA sequence contains a gene encoding each enzyme and is useful for biotechnological production of the polypeptide, and such biotechnological production cannot be performed by only the DNA sequence having a definite length but can be performed in the state where other DNA sequence with a proper length is linked to the 5′-upstream and/or the 3′-downstream of the DNA sequence. Therefore, the term "DNA sequence" in the present invention includes, in addition to those having a definite length (for example, the length in the region of A - B in the corresponding amino acid sequence of Fig. 1), those in the form of a linear DNA strand or a circular DNA strand containing the DNA sequence having a definite length as a member.

One of the typical forms of each DNA sequence according to the present invention is a form of a plasmid which comprises the DNA sequence as a part of a member or a form in which the plasmid is present in a host such as E. coli. The plasmid as one of the preferable existing forms of each DNA sequence according to the present invention is a conjunction of the DNA sequence according to the present invention as a passenger or a foreign gene, a replicable plasmid vector present stably in a host and a promoter (containing ribosome-binding sites in the case of a procaryote). As the plasmid vector and the promoter, an appropriate combination of those which are well-known can be used.

Polypeptides encoded by DNA sequences

As mentioned above, the DNA sequences according to the present invention are respectively specified by the amino acid sequences of the polypeptides encoded thereby. Each of these polypeptides is the one having an amino acid sequence which corresponds substantially to an amino acid sequence in a specific region as described above in Figs. 1 - 6 (for example, from A to B in Fig. 1). Here, in the six (A-B, C-D, E-F, G-H, I-J, K-L) polypeptides shown in Figs. 1-6 (i.e. six enzymes participating in the formation of carotenoids), some of the amino acids can be deleted or substituted or some amino acids can be added or inserted, etc., so long as each polypeptide has the aforementioned enzymatic activity in the relationship of a substrate and a converted substance (a product). This is indicated by the expression "whose amino acid sequence corresponds substantially to ..." in the claims. For example, each polypeptide that first amino acid (Met) has been deleted from each polypeptide shown in Figs. 1 - 6 is included in such deleted polypeptides.

The typical polypeptides having enzymatic activities, respectively, in the present invention are those in the specific regions in Figs. 1 - 6 described above, and the amino acid sequences of these polypeptides have not been known.

Nucleotide sequences of DNA sequences

The DNA sequences encoding the respective enzymes are those having the nucleotide sequences in the aforementioned specific regions in Figs. 1 - 6 (for example, A-B in Fig. 1) or degenerative isomers thereof, or those having the nucleotide sequences corresponding to the aforementioned alteration of the amino acid sequence of respective enzymes or degenerative isomers thereof. The term "degenerative isomer" means DNA sequence which is different only in degenerative codon and can code for the same polypeptide. The preferred embodiments of the DNA sequences according to the present invention are those having at least one stop codon (such as TAA) at the 3′-terminal. The 5′-upstream and/or the 3′-downstream of the DNA sequences according to the present invention may further have a DNA sequence with a certain length as a non-translation region (the initial portion of the 3′-downstream being usually a stop codon such as TAA).

Gene group used for the synthesis of carotenoids

The gene group (the gene cluster in some case) used for the synthesis of carotenoids comprises a plurality of the aforementioned DNA sequences ① - ⑥, whose typical examples are illustrated in the following (1) - (4). Each gene group encodes a plurality of polypeptides of respective enzymes and these enzymes participate in the production reaction of carotenoids to produce them from their substrates.

(1) Gene group used for the synthesis of lycopene

The gene group used for the synthesis of lycopene which is a red carotenoid is DNA sequence comprising the aforementioned DNA sequences ①, ④ and ⑤, and such a gene group includes the one in which respective DNA sequences are present on one DNA strand or on different DNA strands separately or the one which is constructed by the combination of the aforementioned ones according to necessities.

In the case that a plurality of DNA sequences are present on one DNA strand, the arrangement order and direction of the aforementioned DNA sequences ①, ④ and ⑤ may be optional provided that the genetic information is capable of expression, that is to say respective genes in a host are in a state of being transcribed and translated appropriately.

The biosynthetic pathway of lycopene in E. coli is explained as follows: geranylgeranyl pyrophosphate which is a substrate originally present in E. coli is converted into prephytoene pyrophosphate by the enzyme encoded by the DNA sequence ⑤, the prephytoene pyrophosphate is then converted into phytoene by the enzyme encoded by the DNA sequence ①, and the phytoene is further converted into lycopene by the enzyme encoded by the DNA sequence ④ (see Fig. 8).

Lycopene is a carotene whose color is red. Lycopene is a red pigment which is present in a large amount in the fruits of water melon or tomato and has high safety for food. In this connection, the lycopene which was synthesized by the DNA sequences according to the present invention in the experimental

example described below had the same stereochemistry as lycopene present in these plants.

One of the typical existing forms of the gene group of the present invention is a form of a plasmid which comprises the respective DNA sequences containing a stop codon as a member or a form in which the plasmid is present in a host such as E. coli. The plasmid which is one of the preferred existing forms of the gene group according to the present invention comprises a gene group as a passenger or a foreign gene, a replicable plasmid vector present stably in a host and a promoter (containing ribosome-binding sites in the case of a procaryote). As the promoter, in procaryotes such as E. coli or Zymomonas species a promoter which is common to respective DNA sequences can be used, or alternatively respective promoters can be used to the respective DNA sequences. In the case of eucaryotes such as yeast or plant, respective promoters are preferably used to respective DNA sequences.

One of the preferred existing forms of the DNA sequences are described above in the explanation of the DNA sequences ① - ⑥.

(2) Gene group used for the synthesis of β-carotene

The gene group used for the synthesis of β-carotene which is one of yellow-orange carotenoids is a DNA sequence comprising the aforementioned DNA sequences ①, ③, ④ and ⑤. In other words, the gene group used for the synthesis of β-carotene is formed by adding the DNA sequence ③ to a DNA sequence used for the synthesis of lycopene comprising the DNA sequences and ①, ④, and ⑤. The gene group includes the one in which the respective DNA sequences constructing the gene group may be present on one DNA strand or on different DNA strands individually, or the one which is constructed by the combination of the aforementioned ones according to necessities.

In the case that a plurality of DNA sequences are present on one DNA strand, the arrangement order and direction of the aforementioned DNA sequences ①, ③, ④ and ⑤ may be optional provided that the genetic information is capable of expression, that is to say respective genes in a host are in a state of being transcribed and translated appropriately.

The biosynthetic pathway of β-carotene in E. coli is explained as follows: geranylgeranyl pyrophosphate which is a substrate originally present in E. coli is converted into prephytoene pyrophosphate by the enzyme encoded by the DNA sequence ⑤, the prephytoene pyrophosphate is converted into phytoene by the enzyme encoded by the DNA sequence ①, the phytoene is further converted into lycopene by the enzyme encoded by the DNA sequence ④, and the lycopene is further converted into β-carotene by the enzyme encoded by the DNA sequence ③, (see Fig. 8).

β-carotene is a typical carotene whose color is in the spectrum ranging from yellow to orange, and it is an orange pigment which is present in a large amount in the roots of carrot or green leaves of plants and has high safety for food. The utility of β-carotene has already been described in the explanation of related art. In this connection, the β-carotene which was synthesized by the DNA sequence according to the present invention in the experimental example described below had the same stereochemistry as β-carotene present in the roots of carrot or green leaves of plants.

One of the typical existing forms of the gene group and the individual DNA sequences are the same as defined in (1).

(3) Gene group used for the synthesis of zeaxanthin

The gene group used for the synthesis of zeaxanthin which is one of yellow-orange carotenoids is a DNA sequence comprising the aforementioned DNA sequences ①, ③, ④, ⑤ and ⑥. In other words, the DNA sequence used for the synthesis of zeaxanthin is formed by adding the DNA sequence ⑥ to a DNA sequence used for the synthesis of β-carotene comprising the DNA sequences ①, ③, ④, ⑤ . The gene group includes the one in which the respective DNA sequences constructing the gene group are present on one DNA strand or on different DNA strands individually, or the one which is constructed by the combination of the aforementioned ones according to necessities.

In the case that a plurality of DNA sequences are present on one DNA strand, the arrangement order and direction of the aforementioned DNA sequences ①, ③, ④, ⑤ and ⑥ may be optional provided that the genetic information is capable of expression, that is to say respective genes in a host are in a state of being transcribed and translated appropriately.

The biosynthetic pathway of zeaxanthin in E. coli is explained as follows: geranylgeranyl pyrophosphate which is a substrate originally present in E. coli is converted into prephytoene pyrophosphate by the

enzyme encoded by the DNA sequence ⑤ the prephytoene pyrophosphate is converted into phytoene by the enzyme encoded by the DNA sequence ①, the phytoene is then converted into lycopene by the enzyme encoded by the DNA sequence ④, and the lycopene is further converted into β-carotene by the enzyme encoded by the DNA sequence ③, and finally the β- carotene is converted into zeaxanthin by the enzyme encoded by the DNA sequence ⑥ (see Fig. 8).

Zeaxanthin is a xanthophyll whose color is in the spectrum ranging from yellow to orange, and it is an yellow pigment which is present in the seed of maize and has high safety for food. Zeaxanthin is contained in feeds for hen or colored carp and is an important pigment source for coloring them. In this connection, the zeaxanthin which was synthesized by the DNA sequences according to the present invention in the experimental example described below had the same stereochemistry as zeaxanthin described above.

One of the typical existing forms of the gene group and the individual DNA sequences is the same as defined in (1).

(4) Gene group used for the synthesis of zeaxanthin-diglucoside

The gene group used for the synthesis of zeaxanthin-diglucoside which is one of yellow-orange carotenoids is a DNA sequence comprising the aforementioned DNA sequences ① - ⑥. In other words, the gene group used for the synthesis of zeaxanthin-diglucoside is formed by adding the DNA sequence ② to a DNA sequence used for the synthesis of zeaxanthin comprising the DNA sequences ①, ③, ④, ⑤ and ⑥. The gene group includes the one in which the respective DNA sequences constructing the gene group are present on one DNA strand or on different DNA strands individually, or the one which is constructed by the combination of the aforementioned ones according to necessities.

In the case that a plurality of DNA sequences are present on one DNA strand, the arrangement order and direction of the aforementioned DNA sequences ① - ⑥ may be optional provided that the genetic information is capable of expression, that is to say respective genes in a host are in a state of being transcribed and translated appropriately.

One of the typical existing forms of the gene group and the individual DNA sequences is the same as defined in (1).

The biosynthetic pathway of zeaxanthin-diglucoside in E. coli is explained as follows: geranylgeranyl pyrophosphate which is a substrate originally present in E. coli is converted into prephytoene pyrophosphate by the enzyme encoded by the DNA sequence ⑤, the prephytoene pyrophosphate is converted into phytoene by the enzyme encoded by the DNA sequence ①, the phytoene is then converted into lycopene by the enzyme encoded by the DNA sequence ④, and the lycopene is further converted into β-carotene by the enzyme encoded by the DNA sequence ③, the β-carotene is then converted into zeaxanthin by the enzyme encoded by the DNA sequence ⑤, and the zeaxanthin is finally converted into zeaxanthin-diglucoside by the enzyme encoded by the DNA sequence ② (see Fig. 8).

Zeaxanthin-diglucoside is a carotenoid glycoside having a high water solubility and a pigment which is soluble sufficiently in water at room temperature and exhibits clear yellow. Carotenoid pigments are generally hydrophobic and thus limited on their use as natural coloring materials in foods or the like. Therefore, zeaxanthin-diglucoside settles this defect. Zeaxanthin-diglucoside is isolated from edible plant saffron, Croccus sativus (Pure & Appl. Chem., 47, 121-128 (1976)), so that it is thought that its safety for food has been confirmed. Therefore, zeaxanthin-diglucoside is desirable as a yellow natural coloring material of foods or the like. In this connection, there has been heretofore no reports with reference to the isolation of zeaxanthin-diglucoside from microorganisms.

If carotenoid pigments such as lycopene, β-carotene, zeaxanthin and zeaxanthin-diglucoside are intended to be produced, the aforementioned DNA sequences ①, ④ and ⑤, the DNA sequences ①, ③, ④ and ⑤, the DNA sequences ①, ③, ④, ⑤ and ⑥, and the DNA sequences ① - ⑥ are required, respectively, on using E. coli as the host. However, when a host other than E. coli, particularly the one which is capable of producing carotenoids is used, it has a high possibility of containing also carotenoid precursors at further downstream in the biosynthesis, so that all of the aforementioned DNA sequences ①, ④ and ⑤ (for the production of lycopene), all of the DNA sequences ①, ③, ④ and ⑤ (for the production of β-carotene), all of the DNA sequences ①, ③, ④, ⑤ and ⑥ (for the production of zeaxanthin), or all of the DNA sequences ① - ⑥ (for the production of zeaxanthin-diglucoside) are not always required.

That is to say, only the DNA sequence(s) participating in the formation of an aimed carotenoid pigment from a carotenoid precursor present at the furthest downstream in the host may also be used in this case. Thus, when lycopene is intended to be produced as an aimed carotenoid in a host in which phytoene is preliminarily present, it is also possible to use only the DNA sequence ④ among the DNA sequences ①.

④ and ⑤.

It is also possible to make a host to produce, as the aimed carotenoid pigment relating compound, prephytoene pyrophosphate from geranylgeranyl pyrophosphate by using only the DNA sequence ⑤ of the present invention, or phytoene by using the DNA sequences ① and ⑤ of the present invention or, if the host contains prephytoene pyrophosphate, by using only the DNA sequence ① .

Acquirement of DNA sequences

A method for acquiring the DNA sequences ① - ⑥ which contain the nucleotide sequences coding for the amino acid sequences of the respective enzymes is the chemical synthesis of at least a part of their strand by the method of polynucleotide synthesis. However, if it is taken into consideration that a number of amino acids are bonded, it would be more preferable than the chemical synthesis to acquire the DNA sequences from the DNA library of Erwinia uredovora 20D3 ATTC 19321 according to a conventional method in the field of genetic engineering, for example, the hybridization method with a suitable probe.

The individual DNA sequences or the DNA sequence comprising all of these sequences are thus obtained.

Transformant

The aforementioned gene group comprising a plurality of the DNA sequences ① - ⑥ can be constituted by using the DNA sequences obtained as described above. The DNA sequence thus obtained contains genetic informations for making an enzyme participating in the formation of carotenoids, so that it can be introduced into an appropriate host by the biotechnological method to form a transformant and to produce an enzyme and in its turn a carotenoid pigment or a carotenoid pigment relating compound.

(1) Host

Plants and a variety of microorganisms, as far as a suitable host-vector system is present, can be the target of transformation by a vector comprising the aforementioned DNA sequences. However, the host is required to contain geranylgeranyl pyrophosphate which is a substrate compound of an enzyme for starting the carotenoid synthesis with use of the DNA sequences of the present invention, or a compound further downstream from it.

It is known that geranylgeranyl pyrophosphate is synthesized by dimethylallyltransferase which is a common enzyme at the initial stage of the biosynthesis of not only carotenoids but also sterols or terpenes [J. Biochem., 72, 1101-1108 (1972)]. Accordingly, if a cell which cannot synthesize carotenoids can synthesize sterols or terpenes, it probably contains geranylgeranyl pyrophosphate. It is believed that a cell contains at least one of sterols or perpenes.

Therefore, it is believed theoretically that almost all hosts are capable of synthesizing carotenoids by using the DNA sequences of the present invention as far as a suitable host-vector system is present.

As the hosts in which the host-vector system is present, there are mentioned plants such as Nicotiana tabacum, Petunia hybrida and the like, microorganism such as bacteria, for example Escherichia coli, Zymomonas mobilis and the like, and yeasts, for example Saccharomyces cerevisiae and the like.

(2) Transformation

It is confirmed for the first time by the present invention that the genetic informations present on the DNA sequences of the present invention has been expressed in microorganisms. However, the procedures or the methods for making the transformants (and the production of enzymes or in its turn carotenoid pigments or carotenoid pigment relating compounds by the transformants) are per se conventional in the fields of molecular biology, cell biology or genetic manipulation, and thus the procedures other than described below may be performed in accordance with these conventional techniques.

In order to express the gene of the DNA sequences according to the present invention in a host, it is necessary to insert the gene into a vector for introducing it into the host. As the vector used in this stage, there is used all of various known vectors such as pBI121 or the like for plants (Nicotiana tabacum, Petunia

hybrida); pUC19, pACYC184 or the like for E. coli; PZA22 or the like for Zymomonas mobilis (see Japanese Patent Laid-Open Publication No. 228278/87); and YEp13 or the like for yeast.

On the other hand, it is necessary to transcribe the DNA sequence of the present invention onto mRNA in order to express the gene of the DNA sequence in the host. For this purpose, a promoter as a signal for the transcription may be integrated into the 5′-upstream region from the DNA sequence of the present invention. A variety of promoters such as CaMV35S, NOS, TR1′, TR2′ (for plants); lac, Tc^r, CAT, trp (for E. coli); Tc^r, CAT (for Zymomonas mobilis); ADH1, GAL7, PGK, TRP1 (for yeast) and the like are known as for the promoters, and either of these promoters can be used in the present invention.

In the case of procaryote, it is necessary to place ribosome-binding site (SD sequence in E. coli) several base-upstream from the initiation codon (ATG).

In this connection, while the aforementioned manipulation is necessary for producing the enzyme protein, one or more of amino acids may be inserted into or added to the polypeptide which is illustrated in the specific ranges of Figs. 1 - 6 (e.g. the polypeptide A -B illustrated in Fig. 1), one or more of amino acids may be deleted, or replaced, as described above.

The transformation of the host with the plasmid thus obtained can be conducted optionally by an appropriate method which is conventionally used in the fields of genetic manipulation or cell biology. As for the general matters, there can be referred to appropriate publications or reviews; for example as for the transformation of microorganisms, T. Maniatis, E. F. Fritsch and J. Sambrook: "Molecular Cloning A Laboratory Manual", Cold Spring Harbor Laboratory (1982).

The transformant is the same as the host used, in its genotype, phenotype or bacteriological properties but for the new trait derived from the genetic information introduced by the DNA sequence of the present invention (that is, the production of an enzyme participating in the carotenoid formation and the synthesis of carotenoids or the like by the enzyme), the trait derived from the vector used and the deletion of the trait corresponding to the deletion of a part of the genetic information of the vector which might be caused on the recombination of genes. Escherichia coli JM109 (pCAR1) which is an example of the transformant according to the present invention is deposited as FERM BP-2377.

Expression of genetic information/production of carotenoids

The clone of the transformant obtained as described above produces mainly in the transformant an enzyme participating in the carotenoid formation, and a variety of carotenoids or carotenoid pigment relating compounds are synthesized by the enzyme.

Culture or the culturing condition of the transformant is essentially the same as those for the host used.

Carotenoids can be recovered by the methods, for example, illustrated in Experimental Examples 3 and 4 below.

Furthermore, each enzyme protein coded by each DNA sequence of the present invention is produced mainly in the cell in the case of the transformation of E. coli, and it can be recovered by an appropriate method.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1 - 6 illustrate the nucleotide sequences in the DNA sequences ① - ⑥ in coding regions, and the amino acid sequences of proteins to be encoded, respectively,

Fig. 7 illustrates the KpnI-HindIII fragtment which was acquired from Erwinia uredovora 20D3 ATCC 19321 and relates to the biosynthesis of carotenoids, that is the complete nucleotide sequence of the 6918 bp DNA sequence containing the DNA sequences in Figs. 1 - 6, and

Fig. 8 illustrates the function of the polypeptides encoded by the aforementioned DNA sequences ① - ⑥.

Experiments

All of strains used in the following experiments are deposited in ATCC or other deposition organizations and are freely available.

Experimental Example 1: Cloning of a gene cluster participating in the biosynthesis of a yellow pigment (referred to hereinafter as yellow pigment-synthesizing gene cluster)

(1) Preparation of total DNA

Total DNA was prepared from the cells of Erwinia uredovora 20D3 ATCC 19321 which had been proliferated until the early-stationary phase in 100 ml of LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl). Penicillin G (manufactured by Meiji Seika) was added to the culture medium so that it has a concentration of 50 units/ml in the medium before 1 hour of the harvest of the cells. After harvesting the cells by centrifugation, this was washed with the TES buffer (20 mM tris, 10 mM EDTA, 0.1 M NaCl, pH 8), heat treated at 68°C for 15 minutes and suspended in Solution I (50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8) containing 5 mg/ml of lysozyme (manufactured by Seikagaku Kogyo) and 100 µg/ml of RNase A (manufactured by Sigma). The suspension was incubated at 37°C for a period of 30 minutes - 1 hour, and pronase E (manufactured by Kaken Seiyaku) was added so that it had a concentration of 250 µg/ml before incubation at 37°C for 10 minutes. Sodium N-lauroylsarcosine (manufactured by Nacalai tesque) was added so as it had the final concentration of 1%, and the mixture was agitated before incubation at 37°C for several hours. Extraction was conducted several times with phenol/chloroform. While ethanol in volume of 2 equivalents was slowly added, the resulting total DNA was wound around a glass stick, rinsed with 70% ethanol and dissolved in 2 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) to give the total DNA preparation.

(2) Construction of an Escherichia coli cosmid library and acquirement of E. coli transformants producing yellow pigments

Incubation was conducted with 1 unit of restriction enzyme Sau3AI per 50 µl of the total DNA preparation at 37°C for 30 minutes before the inactivation treatment of the restriction enzyme at 68°C for 10 minutes. Many fragments partially digested with Sau3AI were obtained in the neighbourhood of 40 kb under this condition. After the ethanol precipitation of this reaction solution, this half portion was mixed with 2.5 µg of cosmid PJB8 which had been digested with BamHI and treated with alkaline phosphatase and 0.2 µg of a pJB8 SalI-BamHI right arm fragment (smaller fragment) which had been recovered from a gel, and 40 µl of the total amount was subjected to ligation reaction with T4 DNA ligase at 12°C for 2 days. In this connection, the cosmid pJB8 had been previously purchased from Amersham. Restriction enzymes and enzymes used for genetic manipulation were purchased from Boehringer-Mannheim, Takara Shuzo or Wako Pure Chemical Industries. This DNA in which the ligation reaction had been thus performed was used for in vitro packaging with a Gigapack Gold (manufactured by Stratagene, marketed from Funakoshi) to give a large amount of phage particles sufficient for construction of a cosmid library. The phage particles were infected with Escherichia coli DH1 (ATCC 33849). After the cells of E. coli DH1 infected were diluted so as to be 100 colonies per plate, they were plated on a LB plate, cultured at 37°C overnight and further at 30°C for 6 hours or more. As a result, E. coli transformants producing yellow pigments appeared in a proportion of one colony per about 1,100 colonies. These E. coli transformants producing yellow pigments contained plasmids in which 33 - 47 kb Sau3AI partial digestion fragments were inserted into the pJB8.

(3) Location of a yellow pigment-synthesizing gene cluster

A yellow pigment-synthesizing gene cluster was inserted into the pJB8 as the 33 - 47 kb Sau3AI partial digestion fragments. One of these fragments was further subjected to partial digestion with Sau3AI, ligated to the BamHI site of the E. coli vector pUC19 (purchased from Takara Shuzo), and used to transform Escherichia coli JM109 (manufactured by Takara Shuzo). To locate the yellow pigment-synthesizing gene cluster, plasmid DNA's were prepared from 50 E. coli transformants producing yellow pigments which appeared in the LB plate containing ampicillin, and analyzed by agarose gel electrophoresis. As a result, it was found that the smallest inserted fragment was of 8.2 kb. The plasmid containing this 8.2 kb fragment was named as pCAR1 and E. coli JM109 harboring this plasmid was named as Escherichia coli JM109 (pCAR1). This strain produced the same yellow pigments as those of E. uredovora. The 8.2 kb fragment contained a KpnI site in the neighbourhood of the terminal at the lac promoter side and a HindIII site in the neighbourhood at the opposite side. After the 8.2 kb fragment was subjected to double digestion with

KpnI/HindIII (HindIII was partially digested; the 8.2 kb fragment had two HindIII sites), the KpnI-HindIII fragment (6.9 kb) was recovered from a gel and ligated to the KpnI-HindIII site of pUC18 (this hybrid plasmid was named as pCAR15). Upon the transformation of E. coli JM109, the E. coli transformant exhibited yellow and produced the same yellow pigments as those of E. uredovora. Accordingly, it was found out that the genes required for the yellow pigment production was located on the KpnI-HindIII fragment (6.9 kb). That is to say, the fragment carrying the yellow pigment-synthesizing genes was capable of being reduced to a 6.9 kb in size.

Experimental Example 2: Analysis of the yellow pigment-synthesizing gene cluster

(1) Determination of the nucleotide sequence of the yellow pigment-synthesizing gene cluster

The complete nucleotide sequence of the 6.9 kb KpnI-HindIII fragment was determined by the kilo-sequence method using Deletion kit for kilo-sequence (manufactured by Takara Shuzo) and the dideoxy method according to Proc. Natl. Acad. Sci. USA, 74 5463-5467 (1977). As a result, it was found that the KpnI-HindIII fragment containing the yellow pigment-synthesizing genes (DNA strand) was 6918 base pairs (bp) in length and its GC content was 54%. The complete nucleotide sequence was shown in Fig. 7 (a) - (g). The KpnI site is represented by the base number 1.

(2) Elucidation of yellow pigment-synthesizing gene cluster

The HindIII side of the 6918 bp fragment (DNA strand) containing the yellow pigment-synthesizing genes (right terminal side in Fig. 7) was deleted with Deletion kit for kilo-sequence. A hybrid plasmid (designated pCAR25) was constructed by inserting a 1 - 6503 fragment, which was obtained by deletion from the HindIII site to nucleotide position 6504, into pUC19. E. coli JM109 harboring pCAR25 [referred to hereinafter as E. coli (pCAR25)] exhibited yellow and produced the same yellow pigments as those of E. uredovora. Therefore, it was thought that the region from the base number 6504 to 6918 in Fig. 7 was not required for yellow pigment production. The nucleotide sequence in the region from the base number 1 to 6503 in the 6918 bp DNA sequence containing the yellow pigment-synthesizing genes was analyzed. As a result, it was found that there were six open reading frames (ORFs). That is to say, there were an ORF coding for a polypeptide with a molecular weight of 32,583 from the base number 225 to 1130 (referred to as ORF1, which corresponds to A - B in Figs. 1 and 7), an ORF coding for a polypeptide with a molecular weight of 47,241 from the base number 1143 - 2435 (referred to as ORF2, which corresponds to C - D in Figs. 2 and 7), an ORF coding for a polypeptide with a molecular weight of 43,047 from the base number 2422 to 3567 (referred to as ORF3, which corresponds to E - F in Figs. 3 and 7), an ORF coding for a polypeptide with a molecular weight of 55,007 from the base number 3582 to 5057 (referred to as ORF4, which corresponds to G - H in Figs. 4 and 7), an ORF coding for a polypeptide with a molecular weight of 33,050 from the base number 5096 to 5983 (referred to as ORF5, which corresponds to I - J in Figs. 5 and 7), and an ORF coding for a polypeptide with a molecular weight of 19,816 from the base number 6452 to 5928 (referred to as ORF6, which corresponds to K - L in Figs. 6 and 7. Only this ORF6 has the opposite orientation with the others). In this connection, each ORF contained at positions several base-upstream from its initiation codon the SD (Shine-Dalgarno) sequence which is homologous with the 3'-region of 16S ribosomal RNA of E. coli. Thus, it was thought that polypeptides were in fact synthesized in E. coli by these six ORFs. This was confirmed by the following in vitro transcription-translation experiment.

That is to say, the in vitro transcription-translation analysis was carried out with DNA in which the plasmid pCAR25 containing ORF1 - ORF6 had been digested with ScaI and with DNAs in which respective fragments containing respective ORFs (containing the SD sequence) of ORF1 - ORF6 had been digested with appropriate restriction enzymes, isolated, inserted into pUC19 or pUC18 so that it was subjected to transcriptional read-through from a lac promoter, and then digested with ScaI. In this experiment, a Prokaryotic DNA-directed translation kit manufactured by Amersham was used. As a result, it was confirmed that the bands of polypeptides corresponding to the aforementioned respective ORFs were detected as the transcription-translation products.

Moreover, all of six ORFs were necessary for production of the same yellow pigments as those of E. uredovora as described below (Experimental Examples 3, 4 and 5). From these results, ORF1, ORF2, ORF3, ORF4, ORF5 and ORF6 were designated as zexA, zexB, zexC, zexD, zexE and zexF genes,

respectively.

The base numbers in Figs. 1 - 6 were represented on the basis of the KpnI site in Fig. 7 as the base number 1 and correspond to each other. The marks A - L in Figs. 1 - 6 correspond to the marks A - L in Fig. 7. The DNA sequence from K to L in Fig. 6 was that of the complementary strand of the DNA sequence from K to L in Fig. 7. That is to say, the DNA sequence illustrated in Fig. 6 has the opposite orientation in transcription with the DNA sequences in Figs. 1 - 5 in the original DNA sequence (Fig. 7).

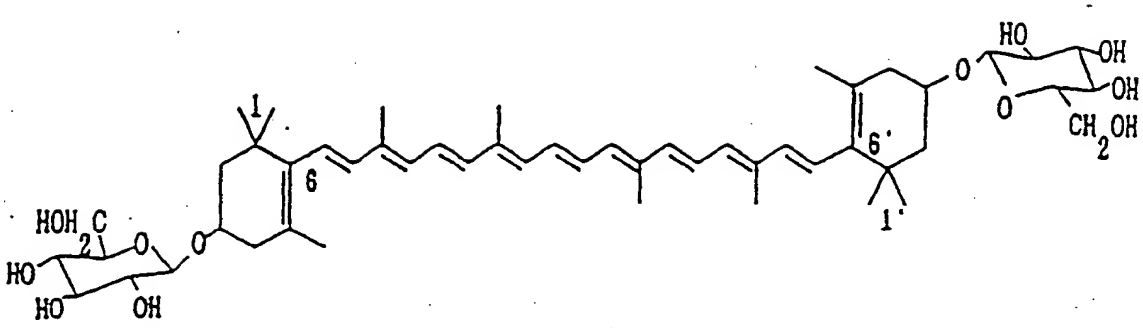
(3) Analysis of homology by the DNA-DNA hybridization method

Total DNA of Erwinia herbicola Eho 10 ATCC 39368 was prepared in the same manner as in Experimental Example 1 (1). A 7.6 kb fragment containing the DNA sequence in Fig. 7 was cut out from the hybrid plasmid pCAR1 by KpnI digestion and labeled with DNA labeling & detection kit nonradioactive (manufactured by Boehringer-Mannheim) according to the DIG-ELISA method to give probe DNA. The homology of total DNAs (intact or KpnI digested) of E. herbicola Eho 10 ATCC 39368 and E. uredovora 20D3 ATCC 19321 with this probe DNA was analyzed by the DNA-DNA hybridization method with the aforementioned DNA labeling & detection kit nonradioactive. As a result, the probe DNA was hybridized strongly with total DNA of the latter E. uredovora 20D3 ATCC 19321, but not at all with total DNA of the former E. herbicola Eho 10 ATCC 39368. Also, the restriction map deduced from the DNA sequence in Fig. 7 was quite different from that reported in J. Bacteriol., 168, 607-612 (1986). It was concluded from the above described results that the DNA sequence in Fig. 7, that is, the DNA sequences useful for the synthesis of carotenoids according to the present invention exhibits no homology with the DNA sequence containing the yellow pigment-synthesizing genes of E. herbicola Eho 10 ATCC 39368.

Experimental Example 3: Analysis of yellow pigments

E. coli (pCAR25) produced the same yellow pigments as those of E. uredovora 20D3 ATCC 19321 and E. herbicola Eho 10 ATCC 39368, and its yield was 5 times higher than those of the former and 6 times higher than those of the latter (per dry weight). The cells harvested from 8 liters of 2 × YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) were extracted once with 1.2 liter of methanol. The methanol extract was evaporated to dryness, dissolved in methanol, and subjected to thin layer chromatography (TLC) with silica gel 60 (Merck) (developed with chloroform : methanol = 4:1). The yellow pigments were separated into 3 spots having Rf values of 0.93, 0.62 and 0.30 by TLC. The yellow (to orange) pigment at the Rf value of 0.30 which was the strongest spot was scraped up from the TLC plate, extracted with a small amount of methanol, loaded on a Sephadex LH-20 column for chromatography [30 cm × 3.0 cm (ø)] and developed and eluted with methanol to give 4 mg of a pure product. The yellow (to orange) pigment obtained was sparingly soluble in organic solvents other than methanol and easily soluble in water, so that it was suggested that it might be a carotenoid glycoside. Such suggestion was also supported from a molecular weight of 892 by FD-MS spectrum (the mass of this pigment was larger than that of zeaxanthin (described hereinafter) by the mass of two glucose). When this substance was hydrolyzed with 1N HCl at 100° C for 10 minutes, zeaxanthin was obtained. Then, acetylation was conducted according to the usual method. That is, the substance was dissolved in 10 ml of pyridine, large excess of acetic anhydride was added, and the mixture was stirred at room temperature and left standing overnight. After the completion of reaction, water was added to the mixture and chloroform extraction was carried out. The chloroform extract was concentrated and loaded on a silica gel column [30 cm × 3.0 cm (ø)] for chromatography to develop and elute with chloroform. Measurement of ^{1}H-NMR gave the spectrum identical with the tetraacetyl derivative of zeaxanthin-β-diglucoside [Helvetica Chimica Acta, 57, 1641-1651 (1974)], so that the substance was identified as zeaxanthin-β-diglucoside (its structure being illustrated below).

The yield was 1.1 mg/g dry weight. The substance had a solubility of at least 2 mg in 100 ml of water and methanol, and water was superior to methanol in solubility of the substance. The substance had low solubilities in chloroform and acetone, and its solubilities were 0.5 mg in 100 ml of these solvents.



Experimental Example 4: Analysis of the metabolic intermediates of carotenoids

(1) Construction of various deletion plasmids

A hybrid plasmid (designated as pCAR16) was constructed by inserting a 1-6009 fragment, which was obtained by deletion to nucleotide position 6010 from the HindIII site (right terminal in Fig. 7) of the 6918 bp fragment containing the yellow pigment-synthesizing genes (DNA strand) (Fig. 7) using Deletion kit for kilo-sequence. pCAR16 contains the genes from zexA to zexE. Various deletion plasmids were constructed, as shown in Table 1, on the basis of the pCAR16 and the aforementioned hybrid plasmid pCAR25 (containing genes from zexA to zexF).

Table 1: Construction of various deletion plasmids

The number within parentheses behind the name of respective restriction enzymes represents the number of base at the initial recognition site of the restriction enzyme. The base numbers correspond to those in Figs. 1 - 6 and Fig. 7. Analysis of the metabolic intermediates of carotenoids was performed using the transformants of E. coli JM109 by various deletion plasmids [referred to hereinafter as E. coli (name of plasmid)].

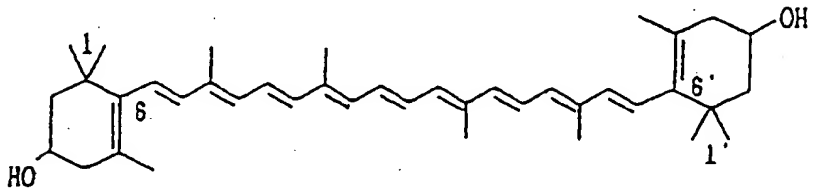
Table 1

Plasmid	Construction method	Genes functioning
pCAR25	See text	zexA zexB zexC zexD zexE zexF
pCAR25delB	Frame shift in BstEII (1235) of pCAR25	zexA zexC zexD zexE zexF
pCAR16	See text	zexA zexB zexC zexD zexE
pCAR16delB	Frame shift in BstEII (1235) of pCAR16	zexA zexC zexD zexE
pCAR16delC	Frame shift in SnaBI (3497) of pCAR16	zexA zexB zexD zexE
pCAR-ADE	Deletion of the BstEII (1235) - SnaBI (3497) fragment from pCAR16	zexA zexD zexE
pCAR-ADEF	Deletion of the BstEII (1235) - SnaBI (3497) fragment from pCAR25	zexA zexD zexE zexF
pCAR25delD	Frame shift in BamHI (3652) of pCAR25	zexA zexB zexC zexE zexF
pCAR-AE	Deletion of the BstEII (1235) - BamHI (3652) fragment from pCAR16	zexA zexE
pCAR-A	Insertion of the KpnI (1) - BstEII (1235) fragment in pUC19	zexA
pCAR-E	Insertion of the Eco52I (4926) - 6009 fragment in pUC19	zexE
pCAR25delE	Frame shift in MluI (5379) of pCAR25	zexA zexB zexC zexD zexF
pCAR25delA	Frame shift in AvaI (995) of pCAR25	zexB zexC zexD zexE zexF
pCAR-CDE	Insertion of the SalI (2295) - 6009 fragment in pUC19	zexC zexD zexE

(2) Identification of zeaxanthin

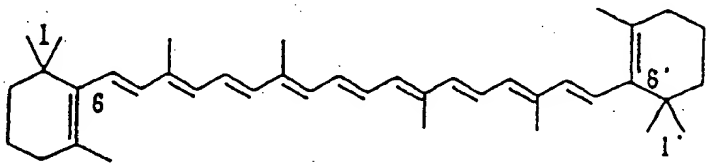
The cells harvested from 3 liters of 2 × YT medium of E. coli (pCAR25delB) (exhibiting orange) were extracted twice with 400 ml portions of acetone at low temperature, concentrated, then extracted with chloroform:methanol (9:1) and evaporated to dryness. This was subjected to silica gel column chromatography [30 cm × 3.0 cm (ø)]. After the column was washed with chloroform, an orange band was eluted with chloroform:methanol (100:1). This pigment was dissolved in ethanol, recrystallized at low temperature to give 8 mg of a pure product. The analysis by its UV-visible absorption, ^{1}H-NMR, ^{13}C-NMR and FD-MS (m/e 568) spectra revealed that this substance had the same structure except for stereochemistry as zeaxanthin (β,β-carotene-3,3′-diol). It was then dissolved in diethyl ether : isopentane ethanol (5:5:2), and the CD spectrum was measured. As a result, it was found that this substance had a 3R,3′R-stereochemistry [Phytochemistry, 27, 3605-3609 (1988)]. Therefore, it was identified as zeaxanthin (β,β-carotene-3R,3′R-

diol), of which the structure is illustrated below. The yield was 2.2 mg/g dry weight. This substance corresponded to the yellow pigment having an Rf value of 0.93 in Experimental Example (1).



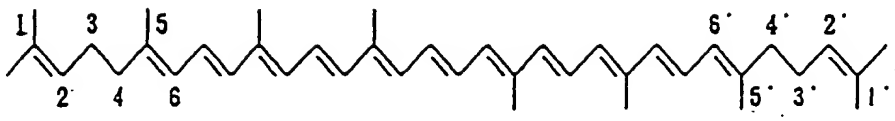
(3) Identification of β-carotene

The cells harvested from 3 liters of LB medium of E. coli (pCAR16) (exhibiting orange) were extracted 3 times with 500 ml portions of cold methanol at low temperature and the methanol extract was further extracted with 1.5 liter of hexane. The hexane layer was concentrated and subjected to silica gel column chromatography [30 cm × 3.0 cm (ø)]. Development and elution were conducted with hexane:ethyl acetate (50:1) to collect an orange band. The orange fraction was concentrated and recrystallized from ethanol to give 8 mg (reduced weight without moisture). This substance was presumed to belong to β-carotene from its UV-visible absorption spectrum, and a molecular weight of 536 by FD-MS spectrum also supported this presumption. Upon comparing this substance with the authentic sample (Sigma) of β-carotene by ^{13}C-NMR spectrum, all of chemical shifts of carbons were identical with each other. Thus, this substance was identified as β-carotene (all-trans-β,β-carotene, of which the structure was illustrated below). It was also confirmed by the similar method that E. coli (pCAR16delB) accumulated the same β-carotene as described above. Its yield was 2.0 mg/g dry weight, which corresponded to 2 -8 times (per dry weight) of the total carotenoid yield in carrot (Kintokininjin) culture cells described in Soshikibaiyou (The Tissue Culture), 13, 379-382 (1987).



(4) Identification of lycopene

The cells harvested from 3 liters of LB medium of E. coli (pCAR16delC) (exhibiting red) were extracted once with 500 ml of cold methanol at low temperature, and the precipitate by centrifugation was extracted again with 1.5 liter of chloroform. The chloroform layer was concentrated and subjected to silica gel chromatography [30 cm × 3.0 cm (ø)]. Development and elution were conducted with hexane:chloroform (1:1) to collect a red band. This fraction was concentrated. This substance was presumed to belong to lycopene from its UV-visible absorption spectrum, and a molecular weight of 536 by FD- MS spectrum also supported this presumption. Upon comparing this substance with the authentic sample (Sigma) of lycopene by ^{1}H-NMR spectrum, all of chemical shifts of hydrogens were identical with each other. When, this substance and the authentic sample were subjected to TLC with silica gel 60 (Merck) [developed with hexane:chloroform (50:1)] and with RP-18 [developed with methanol:chloroform (4:1)], the displacement distances of these samples were completely equal to each other. Thus this substance was identified as lycopene (all-trans-β,β-carotene, of which the structure was illustrated below). It was also confirmed by the similar method that E. coli (pCAR-ADE) and E. coli (pCAR-ADEF) accumulated the same lycopene as described above. The yield of the former was 2.0 mg/g dry weight, which corresponded to 2 times (per dry weight) of the total carotenoid yield in a hyperproduction derivative of carrot (Kintokininjin) culture cells described in Soshikibaiyou (The Tissue Culture), 13, 379-382 (1987).



(5) Identification of phytoene

The cells harvested from 1.5 liter of 2 × YT medium of E. coli (pCAR-AE) were extracted twice with 200 ml portions of acetone and twice with 100 ml portions of hexane, and evaporated to dryness. This was subjected to silica gel chromatography [30 cm × 3.0 cm (ø)]. Development and elution were conducted with hexane:chloroform (1:1) to collect a band which had a strong UV absorption, and it was confirmed to be phytoene by its UV absorption spectrum. It was further subjected to LH-20 column chromatography [30 cm × 3.0 cm (ø)]. Development and elution were conducted with chloroform:methanol (1:1) to give 4 mg of a pure product. The comparison of the ^{1}H-NMR spectrum of this substance with the ^{1}H-NMR spectra of trans- and cis-phytoen (J. Magnetic Resonance, 10, 43-50 (1973)) showed this substance to be a mixture of the trans- and cis-isomers. Isomerization from cis-isomer to trans-isomer hardly occurs, and thus it was judged that such a mixture was produced as a result of trans-cis isomerization in the course of the purification. Therefore, it was concluded that the original phytoene was the trans-type phytoene (all-trans-phytoene, of which the structure was shown below). It was also confirmed by the similar method that E. coli - (pCAR25delD) accumulated the same phytoene as described above.

Experimental Example 5: Identification of carotenoid biosynthesis genes

From the facts that E. coli (pCAR25) produced zeaxanthin-diglucoside and that E. coli (pCAR25delB) harboring a plasmid, in which zexB had been removed from pCAR25, accumulated zeaxanthin, it was found that the zexB gene encoded the glycosylation enzyme which was capable of converting zeaxanthin into zeaxanthin-diglucoside. Similarly, from the fact that E. coli (pCAR16delB) harboring a plasmid, in which zexF had been removed from pCAR25delb, accumulated β-carotene, it was found that the zexF gene encoded the hydroxylation enzyme which was capable of converting β-carotene into zeaxanthin. Similarly, from the fact that the E. coli (pCAR-ADE) harboring a plasmid, in which zexC had been removed from pCAR16delB, accumulated lycopene, it was found that the zexC gene encoded the cyclization enzyme which was capable of converting lycopene into β-carotene. Also, E. coli (pCAR-ADEF) carrying both of the zexA, zexD and zexE genes required for producing lycopene and the zexF gene encoding the hydroxylation enzyme was able to synthesize only lycopene. This demonstrates directly that the hydroxylation reaction in carotenoid biosynthesis occurs after the cyclization reaction. Further, from the facts that E. coli (pCAR-ADE) accumulated lycopene and that E. coli (pCAR-AE) harboring a plasmid, in which the zexD gene had been removed from pCAR-ADE, accumulated phytoene, it was found that the zexD gene encoded the desaturation enzyme which was capable of converting phytoene into lycopene. E. coli (pCAR-A) and E. coli - (pCAR-E) were not able to produce phytoene. It was thought from this result that both of the zexA and zexE genes were required for producing phytoene in E. coli. zexE and zexA were identified as the gene for the conversion of geranylgeranyl pyrophosphate into prephytoene pyrophosphate and that for the conversion of prephytoene pyrophosphate into phytoene, by comparing their putative amino acid sequence with those of crtB and crtE gene products in a photo synthetic bacterium Rhodobacter capsuratus [Mol. Gen. Genet., 216, 254-268 (1988)]. From these analyses described above, all of the six zex genes have been identified and the biosynthetic pathway of carotenoids have also been clear. These results are listed in Fig. 8.

E. coli (pCAR25delE) accumulated no detectable carotenoid intermediate, while E. coli (pCAR25delA) and E. coli (pCAR-CDE) were able to produce a small amount of carotenoids. That is to say, E. coli - (pCAR25delA) and E. coli (pCAR-CDE) produced 4% of zeaxanthin-diglucoside and 2% of β-carotene as

compared with the E. coli (pCAR25) and the E. coli (pCAR16delb), respectively. This result suggests that the reaction from prephytoene pyrophosphate to phytoene may occur non-enzymatically notwithstanding the yield being trace.

As described above, the detailed biosynthetic pathway of carotenoids including general and famous carotenoids such as lycopene, β-carotene and zeaxanthin and water soluble carotenoid such as zeaxanthin-diglucoside were for the first time elucidated, and the gene cluster useful for these biosynthesis was capable of being acquired for the first time. In this connection, lycopene, β-carotene and zeaxanthin which were produced by the genes in the aforementioned Experimental Examples were stereochemically identical with those derived from higher plants [T.W. Goodwin: "Plant Pigments", Academic Press (1988)].

As for zeaxanthin-diglucoside, the isolation from a plant was only reported [Pure & Appl. Chem., 47, 121-128 (1976)], but its isolation from microorganisms has not been reported.

Experimental Example 6: Synthesis of carotenoids in Zymomonas

Zymomonas mobilis is a facultative anaerobic ethanol-producing bacterium. It has a higher ethanol producing rate than that of yeast (Saccharomyces cerevisiae), so that it is preferable as a fuel alcohol-producing bacterium in future. Also, Zymomonas has a special metabolic pathway, Entner-Doudoroff but not glycolytic pathway and cannot produce carotenoids. In order to add further values to this bacterium, the carotenoid biosynthesis genes were introduced into Zymomonas.

The 7.6 kb fragment containing the DNA sequence shown in Fig. 7 was cut out from the hybrid plasmid pCAR1 by KpnI digestion and treated with DNA polymerase I (Klenow enzyme). The fragment thus treated was ligated to the EcoRV site of the cloning vector pZA22 for Zymomonas [see Agric. Biol. Chem., 50, 3201-3203 (1986) and Japanese Patent Laid-Open Publication No. 228278/87] to construct a hybrid plasmid pZACAR1. Also, the 1 -6009 fragment in the DNA sequence in Fig. 7 was cut out from pCAR16 by KpnI/EcoRI digestion and treated with DNA polymerase I (Klenow enzyme). The fragment thus treated was ligated to the EcoRV site of pZA22 to construct a hybrid plasmid pZACAR16. The orientation of the inserted fragments in these plasmids were opposite with the orientation of the Tc^r gene on taking the orientation in Fig. 7 as the normal one. These plasmids were introduced into Z. mobilis NRRL B-14023 by conjugal transfer with the helper plasmid pRK2013 (ATCC 37159) and stably maintained in this strain. Z. mobilis NRRL B-14023 in which pZACAR1 and pZACAR16 had been introduced exhibited yellow, and produced zeaxanthin-diglucoside in an amount of 0.28 mg/g dry weight and β-carotene in an amount of 0.14 mg/g dry weight, respectively. Therefore, carotenoids were successfully produced in Zymomonas by the carotenoid biosynthesis genes according to the present invention.

Deposition of Microorganism

Microorganism relating to the present invention is deposited at Fermentation Research Institute, Japan as follows:

Microorganism	Accession number	Date of deposit
Escherichia coli JM109 (pCAR1)	FERM BP 2377	April 11, 1989

Claims

1. A DNA sequence encoding a polypeptide which has an enzymatic activity for converting prephytoene pyrophosphate into phytoene and whose amino acid sequence corresponds substantially to the amino acid sequence from A to B shown in Figs. 1 (a) and (b).

2. A DNA sequence encoding a polypeptide which has an enzymatic activity for converting zeaxanthin into zeaxanthin-diglucoside and whose amino acid sequence corresponds substantially to the amino acid sequence from C to D shown in Figs. 2 (a) and (b).

3. A DNA sequence encoding a polypeptide which has an enzymatic activity for converting lycopene into β-carotene and whose amino acid sequence corresponds substantially to the amino acid sequence

from E to F shown in Figs. 3 (a) and (b).

4. A DNA sequence encoding a polypeptide which has an enzymatic activity for converting phytoene into lycopene and whose amino acid sequence corresponds substantially to the amino acid sequence from G to H shown in Figs. 4 (a), (b) and (c).

5. A DNA sequence encoding a polypeptide which has an enzymatic activity for converting geranylgeranyl pyrophosphate into prephytoene pyrophosphate and whose amino acid sequence corresponds substantially to the amino acid sequence from I to J shown in Figs. 5 (a) and (b).

6. A DNA sequence encoding a polypeptide which has an enzymatic activity for converting β-carotene into zeaxanthin and whose amino acid sequence corresponds substantially to the amino acid sequence from K to L shown in Fig. 6.

7. A process for producing a carotenoid compound which is selected from the group consisting of prephytoene pyrophosphate, phytoene, lycopene, β-carotene, zeaxanthin and zeaxanthin-diglucoside, which comprises transforming a host with at least one of the DNA sequences according to claims 1-6, and culturing the transformant.

```
   230        240        250        260        270        280
ATGACGGTCTGCGCAAAAAAACACGTTCATCTCACTCGCGATGCTGCGGAGCAGTTACTG
MetThrValCysAlaLysLysHisValHisLeuThrArgAspAlaAlaGluGlnLeuLeu
A

   290        300        310        320        330        340
GCTGATATTGATCGACGCCTTGATCAGTTATTGCCCGTGGAGGGAGAACGGGATGTTGTG
AlaAspIleAspArgArgLeuAspGlnLeuLeuProValGluGlyGluArgAspValVal

   350        360        370        380        390        400
GGTGCCGCGATGCGTGAAGGTGCGCTGGCACCGGGAAAACGTATTCGCCCCATGTTGCTG
GlyAlaAlaMetArgGluGlyAlaLeuAlaProGlyLysArgIleArgProMetLeuLeu

   410        420        430        440        450        460
TTGCTGACCGCCCGCGATCTGGGTTGCGCTGTCAGCCATGACGGATTACTGGATTTGGCC
LeuLeuThrAlaArgAspLeuGlyCysAlaValSerHisAspGlyLeuLeuAspLeuAla

   470        480        490        500        510        520
TGTGCGGTGGAAATGGTCCACGCGGCTTCGCTGATCCTTGACGATATGCCCTGCATGGAC
CysAlaValGluMetValHisAlaAlaSerLeuIleLeuAspAspMetProCysMetAsp

   530        540        550        560        570        580
GATGCGAAGCTGCGGCGCGGACGCCCTACCATTCATTCTCATTACGGAGAGCATGTGGCA
AspAlaLysLeuArgArgGlyArgProThrIleHisSerHisTyrGlyGluHisValAla

   590        600        610        620        630        640
ATACTGGCGGCGGTTGCCTTGCTGAGTAAAGCCTTTGGCGTAATTGCCGATGCAGATGGC
IleLeuAlaAlaValAlaLeuLeuSerLysAlaPheGlyValIleAlaAspAlaAspGly

   650        660        670        680        690        700
CTCACGCCGCTGGCAAAAAATCGGGCGGTTTCTGAACTGTCAAACGCCATCGGCATGCAA
LeuThrProLeuAlaLysAsnArgAlaValSerGluLeuSerAsnAlaIleGlyMetGln

   710        720        730        740        750        760
GGATTGGTTCAGGGTCAGTTCAAGGATCTGTCTGAAGGGGATAAGCCGCGCAGCGCTGAA
GlyLeuValGlnGlyGlnPheLysAspLeuSerGluGlyAspLysProArgSerAlaGlu

   770        780        790        800        810        820
GCTATTTTGATGACGAATCACTTTAAAACCAGCACGCTGTTTTGTGCCTCCATGCAGATG
AlaIleLeuMetThrAsnHisPheLysThrSerThrLeuPheCysAlaSerMetGlnMet

   830        840        850        860        870        880
GCCTCGATTGTTGCGAATGCCTCCAGCGAAGCGCGTGATTGCCTGCATCGTTTTTCACTT
AlaSerIleValAlaAsnAlaSerSerGluAlaArgAspCysLeuHisArgPheSerLeu
```

FIG. 1 (a)

```
   890        900        910        920        930        940
GATCTTGGTCAGGCATTTCAACTGCTGGACGATTTGACCGATGGCATGACCGACACCGGT
AspLeuGlyGlnAlaPheGlnLeuLeuAspAspLeuThrAspGlyMetThrAspThrGly

   950        960        970        980        990       1000
AAGGATAGCAATCAGGACGCCGGTAAATCGACGCTGGTCAATCTGTTAGGCCCGAGGGCG
LysAspSerAsnGlnAspAlaGlyLysSerThrLeuValAsnLeuLeuGlyProArgAla

  1010       1020       1030       1040       1050       1060
GTTGAAGAACGTCTGAGACAACATCTTCAGCTTGCCAGTGAGCATCTCTCTGCGGCCTGC
ValGluGluArgLeuArgGlnHisLeuGlnLeuAlaSerGluHisLeuSerAlaAlaCys

  1070       1080       1090       1100       1110       1120
CAACACGGGCACGCCACTCAACATTTTATTCAGGCCTGGTTTGACAAAAAACTCGCTGCC
GlnHisGlyHisAlaThrGlnHisPheIleGlnAlaTrpPheAspLysLysLeuAlaAla

  1130
GTCAGTTAA
ValSer***
```

B

FIG. 1 (b)

```
    1150       1160       1170       1180       1190       1200
ATGAGCCATTTCGCGGCGATCGCACCGCCTTTTTACAGCCATGTTCGCGCATTACAGAAT
MetSerHisPheAlaAlaIleAlaProProPheTyrSerHisValArgAlaLeuGlnAsn
C

    1210       1220       1230       1240       1250       1260
CTCGCTCAGGAACTGGTCGCGCGCGGTCATCGGGTGACCTTTATTCAGCAATACGATATT
LeuAlaGlnGluLeuValAlaArgGlyHisArgValThrPheIleGlnGlnTyrAspIle

    1270       1280       1290       1300       1310       1320
AAACACTTGATCGATAGCGAAACCATTGGATTTCATTCCGTCGGGACAGACAGCCATCCC
LysHisLeuIleAspSerGluThrIleGlyPheHisSerValGlyThrAspSerHisPro

    1330       1340       1350       1360       1370       1380
CCCGGCGCGTTAACGCGCGTGCTACACCTGGCGGCTCATCCTCTGGGGCCGTCAATGCTG
ProGlyAlaLeuThrArgValLeuHisLeuAlaAlaHisProLeuGlyProSerMetLeu

    1390       1400       1410       1420       1430       1440
AAGCTCATCAATGAAATGGCGCGCACCACCGATATGCTGTGCCGCGAACTCCCCCAGGCA
LysLeuIleAsnGluMetAlaArgThrThrAspMetLeuCysArgGluLeuProGlnAla

    1450       1460       1470       1480       1490       1500
TTTAACGATCTGGCCGTCGATGGCGTCATTGTTGATCAAATGGAACCGGCAGGCGCGCTC
PheAsnAspLeuAlaValAspGlyValIleValAspGlnMetGluProAlaGlyAlaLeu

    1510       1520       1530       1540       1550       1560
GTTGCTGAAGCACTGGGACTGCCGTTTATCTCTGTCGCCTGCGCGCTGCCTCTCAATCGT
ValAlaGluAlaLeuGlyLeuProPheIleSerValAlaCysAlaLeuProLeuAsnArg

    1570       1580       1590       1600       1610       1620
GAACCGGATATGCCCCTGGCGGTTATGCCTTTCGAATACGGGACCAGCGACGCGGCTCGC
GluProAspMetProLeuAlaValMetProPheGluTyrGlyThrSerAspAlaAlaArg

    1630       1640       1650       1660       1670       1680
GAACGTTATGCCGCCAGTGAAAAAATTTATGACTGGCTAATGCGTCGTCATGACCGTGTC
GluArgTyrAlaAlaSerGluLysIleTyrAspTrpLeuMetArgArgHisAspArgVal

    1690       1700       1710       1720       1730       1740
ATTGCCGAACACAGCCACAGAATGGGCTTAGCCCCCCGGCAAAAGCTTCACCAGTGTTTT
IleAlaGluHisSerHisArgMetGlyLeuAlaProArgGlnLysLeuHisGlnCysPhe

    1750       1760       1770       1780       1790       1800
TCGCCACTGGCGCAAATCAGCCAGCTTGTTCCTGAACTGGATTTTCCCCGCAAAGCGTTA
SerProLeuAlaGlnIleSerGlnLeuValProGluLeuAspPheProArgLysAlaLeu
```

FIG. 2 (a)

```
    1810       1820       1830       1840       1850       1860
CCGGCTTGTTTTCATGCCGTCGGGCCTCTGCGCGAAACGCACGCACCGTCAACGTCTTCA
ProAlaCysPheHisAlaValGlyProLeuArgGluThrHisAlaProSerThrSerSer

    1870       1880       1890       1900       1910       1920
TCCCGTTATTTTACATCCTCAGAAAAACCCCGGATTTTCGCCTCGCTGGGCACGCTTCAG
SerArgTyrPheThrSerSerGluLysProArgIlePheAlaSerLeuGlyThrLeuGln

    1930       1940       1950       1960       1970       1980
GGACACCGTTATGGGCTGTTTAAAACGATAGTGAAAGCCTGTGAAGAAATTGACGGTCAG
GlyHisArgTyrGlyLeuPheLysThrIleValLysAlaCysGluGluIleAspGlyGln

    1990       2000       2010       2020       2030       2040
CTCCTGTTAGCCCACTGTGGTCGTCTTACGGACTCTCAGTGTGAAGAGCTGGCGCGAAGC
LeuLeuLeuAlaHisCysGlyArgLeuThrAspSerGlnCysGluGluLeuAlaArgSer

    2050       2060       2070       2080       2090       2100
CGTCATACACAGGTGGTGGATTTTGCCGATCAGTCAGCCGCGCTGTCTCAGGCGCAGCTG
ArgHisThrGlnValValAspPheAlaAspGlnSerAlaAlaLeuSerGlnAlaGlnLeu

    2110       2120       2130       2140       2150       2160
GCGATCACCCACGGCGGCATGAATACGGTACTGGACGCGATTAATTACCGGACGCCCCTT
AlaIleThrHisGlyGlyMetAsnThrValLeuAspAlaIleAsnTyrArgThrProLeu

    2170       2180       2190       2200       2210       2220
TTAGCGCTTCCGCTGGCCTTTGATCAGCCCGGCGTCGCGTCACGCATCGTTTATCACGGC
LeuAlaLeuProLeuAlaPheAspGlnProGlyValAlaSerArgIleValTyrHisGly

    2230       2240       2250       2260       2270       2280
ATCGGCAAGCGTGCTTCCCGCTTTACCACCAGCCATGCTTTGGCTCGTCAGATGCGTTCA
IleGlyLysArgAlaSerArgPheThrThrSerHisAlaLeuAlaArgGlnMetArgSer

    2290       2300       2310       2320       2330       2340
TTGCTGACCAACGTCGACTTTCAGCAGCGCATGGCGAAAATCCAGACAGCCCTTCGTTTG
LeuLeuThrAsnValAspPheGlnGlnArgMetAlaLysIleGlnThrAlaLeuArgLeu

    2350       2360       2370       2380       2390       2400
GCAGGGGGCACCATGGCCGCTGCCGATATCATTGAGCAGGTTATGTGCACCGGTCAGCCT
AlaGlyGlyThrMetAlaAlaAlaAspIleIleGluGlnValMetCysThrGlyGlnPro

                                        D
                                        ↓
    2410       2420       2430
GTCTTAAGTGGGAGCGGCTATGCAACCGCATTATGA
ValLeuSerGlySerGlyTyrAlaThrAlaLeu***
```

FIG. 2 (b)

```
     2430       2440       2450       2460       2470       2480
ATGCAACCGCATTATGATCTGATTCTCGTGGGGGCTGGACTCGCGAATGGCCTTATCGCC
MetGlnProHisTyrAspLeuIleLeuValGlyAlaGlyLeuAlaAsnGlyLeuIleAla
E
     2490       2500       2510       2520       2530       2540
CTGCGTCTTCAGCAGCAGCAACCTGATATGCGTATTTTGCTTATCGACGCCGCACCCCAG
LeuArgLeuGlnGlnGlnGlnProAspMetArgIleLeuLeuIleAspAlaAlaProGln

     2550       2560       2570       2580       2590       2600
GCGGGCGGGAATCATACGTGGTCATTTCACCACGATGATTTGACTGAGAGCCAACATCGT
AlaGlyGlyAsnHisThrTrpSerPheHisHisAspAspLeuThrGluSerGlnHisArg

     2610       2620       2630       2640       2650       2660
TGGATAGCTCCGCTGGTGGTTCATCACTGGCCCGACTATCAGGTACGCTTTCCCACACGC
TrpIleAlaProLeuValValHisHisTrpProAspTyrGlnValArgPheProThrArg

     2670       2680       2690       2700       2710       2720
CGTCGTAAGCTGAACAGCGGCTACTTTTGTATTACTTCTCAGCGTTTCGCTGAGGTTTTA
ArgArgLysLeuAsnSerGlyTyrPheCysIleThrSerGlnArgPheAlaGluValLeu

     2730       2740       2750       2760       2770       2780
CAGCGACAGTTTGGCCCGCACTTGTGGATGGATACCGCGGTCGCAGAGGTTAATGCGGAA
GlnArgGlnPheGlyProHisLeuTrpMetAspThrAlaValAlaGluValAsnAlaGlu

     2790       2800       2810       2820       2830       2840
TCTGTTCGGTTGAAAAAGGGTCAGGTTATCGGTGCCCGCGCGGTGATTGACGGGCGGGGT
SerValArgLeuLysLysGlyGlnValIleGlyAlaArgAlaValIleAspGlyArgGly

     2850       2860       2870       2880       2890       2900
TATGCGGCAAATTCAGCACTGAGCGTGGGCTTCCAGGCGTTTATTGGCCAGGAATGGCGA
TyrAlaAlaAsnSerAlaLeuSerValGlyPheGlnAlaPheIleGlyGlnGluTrpArg

     2910       2920       2930       2940       2950       2960
TTGAGCCACCCGCATGGTTTATCGTCTCCCATTATCATGGATGCCACGGTCGATCAGCAA
LeuSerHisProHisGlyLeuSerSerProIleIleMetAspAlaThrValAspGlnGln

     2970       2980       2990       3000       3010       3020
AATGGTTATCGCTTCGTGTACAGCCTGCCGCTCTCGCCGACCAGATTGTTAATTGAAGAC
AsnGlyTyrArgPheValTyrSerLeuProLeuSerProThrArgLeuLeuIleGluAsp

     3030       3040       3050       3060       3070       3080
ACGCACTATATTGATAATGCGACATTAGATCCTGAATGCGCGCGGCAAAATATTTGCGAC
ThrHisTyrIleAspAsnAlaThrLeuAspProGluCysAlaArgGlnAsnIleCysAsp
```

FIG. 3 (a)

```
     3090       3100       3110       3120       3130       3140
TATGCCGCGCAACAGGGTTGGCAGCTTCAGACACTGCTGCGAGAAGAACAGGGCGCCTTA
TyrAlaAlaGlnGlnGlyTrpGlnLeuGlnThrLeuLeuArgGluGluGlnGlyAlaLeu

     3150       3160       3170       3180       3190       3200
CCCATTACTCTGTCGGGCAATGCCGACGCATTCTGGCAGCAGCGCCCCCTGGCCTGTAGT
ProIleThrLeuSerGlyAsnAlaAspAlaPheTrpGlnGlnArgProLeuAlaCysSer

     3210       3220       3230       3240       3250       3260
GGATTACGTGCCGGTCTGTTCCATCCTACCACCGGCTATTCACTGCCGCTGGCGGTTGCC
GlyLeuArgAlaGlyLeuPheHisProThrThrGlyTyrSerLeuProLeuAlaValAla

     3270       3280       3290       3300       3310       3320
GTGGCCGACCGCCTGAGTGCACTTGATGTCTTTACGTCGGCCTCAATTCACCATGCCATT
ValAlaAspArgLeuSerAlaLeuAspValPheThrSerAlaSerIleHisHisAlaIle

     3330       3340       3350       3360       3370       3380
ACGCATTTTGCCCGCGAGCGCTGGCAGCAGCAGGGCTTTTTCCGCATGCTGAATCGCATG
ThrHisPheAlaArgGluArgTrpGlnGlnGlnGlyPhePheArgMetLeuAsnArgMet

     3390       3400       3410       3420       3430       3440
CTGTTTTTAGCCGGACCCGCCGATTCACGCTGGCGGGTTATGCAGCGTTTTTATGGTTTA
LeuPheLeuAlaGlyProAlaAspSerArgTrpArgValMetGlnArgPheTyrGlyLeu

     3450       3460       3470       3480       3490       3500
CCTGAAGATTTAATTGCCCGTTTTTATGCGGGAAAACTCACGCTGACCGATCGGCTACGT
ProGluAspLeuIleAlaArgPheTyrAlaGlyLysLeuThrLeuThrAspArgLeuArg

     3510       3520       3530       3540       3550       3560
ATTCTGAGCGGCAAGCCGCCTGTTCCGGTATTAGCAGCATTGCAAGCCATTATGACGACT
IleLeuSerGlyLysProProValProValLeuAlaAlaLeuGlnAlaIleMetThrThr

     3570
CATCGTTAA
HisArg***
```

F

FIG. 3 (b)

```
      3590       3600       3610       3620       3630       3640
ATGAAACCAACTACGGTAATTGGTGCAGGCTTCGGTGGCCTGGCACTGGCAATTCGTCTA
MetLysProThrThrValIleGlyAlaGlyPheGlyGlyLeuAlaLeuAlaIleArgLeu

G
      3650       3660       3670       3680       3690       3700
CAAGCTGCGGGGATCCCCGTCTTACTGCTTGAACAACGTGATAAACCCGGCGGTCGGGCT
GlnAlaAlaGlyIleProValLeuLeuGluGlnArgAspLysProGlyGlyArgAla

      3710       3720       3730       3740       3750       3760
TATGTCTACGAGGATCAGGGGTTTACCTTTGATGCAGGCCCGACGGTTATCACCGATCCC
TyrValTyrGluAspGlnGlyPheThrPheAspAlaGlyProThrValIleThrAspPro

      3770       3780       3790       3800       3810       3820
AGTGCCATTGAAGAACTGTTTGCACTGGCAGGAAAACAGTTAAAAGAGTATGTCGAACTG
SerAlaIleGluGluLeuPheAlaLeuAlaGlyLysGlnLeuLysGluTyrValGluLeu

      3830       3840       3850       3860       3870       3880
CTGCCGGTTACGCCGTTTTACCGCCTGTGTTGGGAGTCAGGGAAGGTCTTTAATTACGAT
LeuProValThrProPheTyrArgLeuCysTrpGluSerGlyLysValPheAsnTyrAsp

      3890       3900       3910       3920       3930       3940
AACGATCAAACCCGGCTCGAAGCGCAGATTCAGCAGTTTAATCCCCGCGATGTCGAAGGT
AsnAspGlnThrArgLeuGluAlaGlnIleGlnGlnPheAsnProArgAspValGluGly

      3950       3960       3970       3980       3990       4000
TATCGTCAGTTTCTGGACTATTCACGCGCGGTGTTTAAAGAAGGCTATCTAAAGCTCGGT
TyrArgGlnPheLeuAspTyrSerArgAlaValPheLysGluGlyTyrLeuLysLeuGly

      4010       4020       4030       4040       4050       4060
ACTGTCCCTTTTTTATCGTTCAGAGACATGCTTCGCGCCGCACCTCAACTGGCGAAACTG
ThrValProPheLeuSerPheArgAspMetLeuArgAlaAlaProGlnLeuAlaLysLeu

      4070       4080       4090       4100       4110       4120
CAGGCATGGAGAAGCGTTTACAGTAAGGTTGCCAGTTACATCGAAGATGAACATCTGCGC
GlnAlaTrpArgSerValTyrSerLysValAlaSerTyrIleGluAspGluHisLeuArg

      4130       4140       4150       4160       4170       4180
CAGGCGTTTTCTTTCCACTCGCTGTTGGTGGGCGGCAATCCCTTCGCCACCTCATCCATT
GlnAlaPheSerPheHisSerLeuLeuValGlyGlyAsnProPheAlaThrSerSerIle

      4190       4200       4210       4220       4230       4240
TATACGTTGATACACGCGCTGGAGCGTGAGTGGGGCGTCTGGTTTCCGCGTGGCGGCACC
TyrThrLeuIleHisAlaLeuGluArgGluTrpGlyValTrpPheProArgGlyGlyThr
```

FIG. 4 (a)

```
     4250       4260       4270       4280       4290       4300
GGCGCATTAGTTCAGGGGATGATAAAGCTGTTTCAGGATCTGGGTGGCGAAGTCGTGTTA
GlyAlaLeuValGlnGlyMetIleLysLeuPheGlnAspLeuGlyGlyGluValValLeu

     4310       4320       4330       4340       4350       4360
AACGCCAGAGTCAGCCATATGGAAACGACAGGAAACAAGATTGAAGCCGTGCATTTAGAG
AsnAlaArgValSerHisMetGluThrThrGlyAsnLysIleGluAlaValHisLeuGlu

     4370       4380       4390       4400       4410       4420
GACGGTCGCAGGTTCCTGACGCAAGCCGTCGCGTCAAATGCAGATGTGGTTCATACCTAT
AspGlyArgArgPheLeuThrGlnAlaValAlaSerAsnAlaAspValValHisThrTyr

     4430       4440       4450       4460       4470       4480
CGCGACCTGTTAAGCCAGCACCCTGCCGCGGTTAAGCAGTCCAACAAACTGCAGACTAAG
ArgAspLeuLeuSerGlnHisProAlaAlaValLysGlnSerAsnLysLeuGlnThrLys

     4490       4500       4510       4520       4530       4540
CGCATGAGTAACTCTCTGTTTGTGCTCTATTTTGGTTTGAATCACCATCATGATCAGCTC
ArgMetSerAsnSerLeuPheValLeuTyrPheGlyLeuAsnHisHisHisAspGlnLeu

     4550       4560       4570       4580       4590       4600
GCGCATCACACGGTTTGTTTCGGCCCGCGTTACCGCGAGCTGATTGACGAAATTTTTAAT
AlaHisHisThrValCysPheGlyProArgTyrArgGluLeuIleAspGluIlePheAsn

     4610       4620       4630       4640       4650       4660
CATGATGGCCTCGCAGAGGACTTCTCACTTTATCTGCACGCGCCCTGTGTCACGGATTCG
HisAspGlyLeuAlaGluAspPheSerLeuTyrLeuHisAlaProCysValThrAspSer

     4670       4680       4690       4700       4710       4720
TCACTGGCGCCTGAAGGTTGCGGCAGTTACTATGTGTTGGCGCCGGTGCCGCATTTAGGC
SerLeuAlaProGluGlyCysGlySerTyrTyrValLeuAlaProValProHisLeuGly

     4730       4740       4750       4760       4770       4780
ACCGCGAACCTCGACTGGACGGTTGAGGGGCCAAAACTACGCGACCGTATTTTTGCGTAC
ThrAlaAsnLeuAspTrpThrValGluGlyProLysLeuArgAspArgIlePheAlaTyr

     4790       4800       4810       4820       4830       4840
CTTGAGCAGCATTACATGCCTGGCTTACGGAGTCAGCTGGTCACGCACCGGATGTTTACG
LeuGluGlnHisTyrMetProGlyLeuArgSerGlnLeuValThrHisArgMetPheThr

     4850       4860       4870       4880       4890       4900
CCGTTTGATTTTCGCGACCAGCTTAATGCCTATCATGGCTCAGCCTTTTCTGTGGAGCCC
ProPheAspPheArgAspGlnLeuAsnAlaTyrHisGlySerAlaPheSerValGluPro
```

FIG. 4 (b)

```
     4910       4920       4930       4940       4950       4960
GTTCTTACCCAGAGCGCCTGGTTTCGGCCGCATAACCGCGATAAAACCATTACTAATCTC
ValLeuThrGlnSerAlaTrpPheArgProHisAsnArgAspLysThrIleThrAsnLeu

     4970       4980       4990       5000       5010       5020
TACCTGGTCGGCGCAGGCACGCATCCCGGCGCAGGCATTCCTGGCGTCATCGGCTCGGCA
TyrLeuValGlyAlaGlyThrHisProGlyAlaGlyIleProGlyValIleGlySerAla

     5030       5040       5050       5060
AAAGCGACAGCAGGTTTGATGCTGGAGGATCTGATTTGA
LysAlaThrAlaGlyLeuMetLeuGluAspLeuIle***
                                    ↑
                                    H
```

FIG. 4 (c)

```
 5100       5110       5120       5130       5140       5150
ATGGCAGTTGGCTCGAAAAGTTTTGCGACAGCCTCAAAGTTATTTGATGCAAAAACCCGG
MetAlaValGlySerLysSerPheAlaThrAlaSerLysLeuPheAspAlaLysThrArg

 5160       5170       5180       5190       5200       5210
CGCAGCGTACTGATGCTCTACGCCTGGTGCCGCCATTGTGACGATGTTATTGACGATCAG
ArgSerValLeuMetLeuTyrAlaTrpCysArgHisCysAspAspValIleAspAspGln

 5220       5230       5240       5250       5260       5270
ACGCTGGGCTTTCAGGCCCGGCAGCCTGCCTTACAAACGCCCGAACAACGTCTGATGCAA
ThrLeuGlyPheGlnAlaArgGlnProAlaLeuGlnThrProGluGlnArgLeuMetGln

 5280       5290       5300       5310       5320       5330
CTTGAGATGAAAACGCGCCAGGCCTATGCAGGATCGCAGATGCACGAACCGGCGTTTGCG
LeuGluMetLysThrArgGlnAlaTyrAlaGlySerGlnMetHisGluProAlaPheAla

 5340       5350       5360       5370       5380       5390
GCTTTTCAGGAAGTGGCTATGGCTCATGATATCGCCCCGGCTTACGCGTTTGATCATCTG
AlaPheGlnGluValAlaMetAlaHisAspIleAlaProAlaTyrAlaPheAspHisLeu

 5400       5410       5420       5430       5440       5450
GAAGGCTTCGCCATGGATGTACGCGAAGCGCAATACAGCCAACTGGATGATACGCTGCGC
GluGlyPheAlaMetAspValArgGluAlaGlnTyrSerGlnLeuAspAspThrLeuArg

 5460       5470       5480       5490       5500       5510
TATTGCTATCACGTTGCAGGCGTTGTCGGCTTGATGATGGCGCAAATCATGGGCGTGCGG
TyrCysTyrHisValAlaGlyValValGlyLeuMetMetAlaGlnIleMetGlyValArg

 5520       5530       5540       5550       5560       5570
GATAACGCCACGCTGGACCGCGCCTGTGACCTTGGGCTGGCATTTCAGTTGACCAATATT
AspAsnAlaThrLeuAspArgAlaCysAspLeuGlyLeuAlaPheGlnLeuThrAsnIle

 5580       5590       5600       5610       5620       5630
GCTCGCGATATTGTGGACGATGCGCATGCGGGCCGCTGTTATCTGCCGGCAAGCTGGCTG
AlaArgAspIleValAspAspAlaHisAlaGlyArgCysTyrLeuProAlaSerTrpLeu

 5640       5650       5660       5670       5680       5690
GAGCATGAAGGTCTGAACAAAGAGAATTATGCGGCACCTGAAAACCGTCAGGCGCTGAGC
GluHisGluGlyLeuAsnLysGluAsnTyrAlaAlaProGluAsnArgGlnAlaLeuSer

 5700       5710       5720       5730       5740       5750
CGTATCGCCCCGTCGTTTGGTGCAGGAAGCAGAACCTTACTATTTGTCTGCCACAGCCGGC
ArgIleAlaArgArgLeuValGlnGluAlaGluProTyrTyrLeuSerAlaThrAlaGly
```

FIG. 5 (a)

```
 5760       5770       5780       5790       5800       5810
CTGGCAGGGTTGCCCCTGCGTTCCGCCTGGGCAATCGCTACGGCGAAGCAGGTTTACCGG
LeuAlaGlyLeuProLeuArgSerAlaTrpAlaIleAlaThrAlaLysGlnValTyrArg

 5820       5830       5840       5850       5860       5870
AAAATAGGTGTCAAAGTTGAACAGGCCGGTCAGCAAGCCTGGGATCAGCGGCAGTCAACG
LysIleGlyValLysValGluGlnAlaGlyGlnGlnAlaTrpAspGlnArgGlnSerThr

 5880       5890       5900       5910       5920       5930
ACCACGCCCGAAAAATTAACGCTGCTGCTGGCCGCCTCTGGTCAGGCCCTTACTTCCCGG
ThrThrProGluLysLeuThrLeuLeuLeuAlaAlaSerGlyGlnAlaLeuThrSerArg

 5940       5950       5960       5970       5980
ATGCGGGCTCATCCTCCCCGCCCTGCGCATCTCTGGCAGCGCCCGCTCTAG
MetArgAlaHisProProArgProAlaHisLeuTrpGlnArgProLeu***
                                                  ↑
                                                  J
```

FIG. 5 (b)

```
6452
   ATGTTGTGGATTTGGAATGCCCTGATCGTTTTCGTTACCGTGATTGGCATGGAAGTGATT
   MetLeuTrpIleTrpAsnAlaLeuIleValPheValThrValIleGlyMetGluValIle
   ↑
   K
   GCTGCACTGGCACACAAATACATCATGCACGGCTGGGGTTGGGGATGGCATCTTTCACAT
   AlaAlaLeuAlaHisLysTyrIleMetHisGlyTrpGlyTrpGlyTrpHisLeuSerHis

   CATGAACCGCGTAAAGGTGCGTTTGAAGTTAACGATCTTTATGCCGTGGTTTTTGCTGCA
   HisGluProArgLysGlyAlaPheGluValAsnAspLeuTyrAlaValValPheAlaAla

   TTATCGATCCTGCTGATTTATCTGGGCAGTACAGGAATGTGGCCGCTCCAGTGGATTGGC
   LeuSerIleLeuLeuIleTyrLeuGlySerThrGlyMetTrpProLeuGlnTrpIleGly

   GCAGGTATGACGGCGTATGGATTACTCTATTTTATGGTGCACGACGGGCTGGTGCATCAA
   AlaGlyMetThrAlaTyrGlyLeuLeuTyrPheMetValHisAspGlyLeuValHisGln

   CGTTGGCCATTCCGCTATATTCCACGCAAGGGCTACCTCAAACGGTTGTATATGGCGCAC
   ArgTrpProPheArgTyrIleProArgLysGlyTyrLeuLysArgLeuTyrMetAlaHis

   CGTATGCATCACGCCGTCAGGGGCAAAGAAGGTTGTGTTTCTTTTGGCTTCCTCTATGCG
   ArgMetHisHisAlaValArgGlyLysGluGlyCysValSerPheGlyPheLeuTyrAla

   CCGCCCCTGTCAAAACTTCAGGCGACGCTCCGGGAAAGACATGGCGCTAGAGCGGGCGCT
   ProProLeuSerLysLeuGlnAlaThrLeuArgGluArgHisGlyAlaArgAlaGlyAla

                                                          5925
   GCCAGAGATGCGCAGGGCGGGGAGGATGAGCCCGCATCCGGGAAGTAA
   AlaArgAspAlaGlnGlyGlyGluAspGluProAlaSerGlyLys***
                                                  ↑
                                                  L
```

FIG. 6

```
1          10          20          30          40          50
GGTACCGCAC GGTCTGCCAA TCCGACGGAG GTTTATGAAT TTTCCACCTT TTCCACAAGC

          70          80          90         100         110
TCAACTAGTA TTAACGATGT GGATTTAGCA AAAAAAACCT GTAACCCTAA ATGTAAAATA

         130         140         150         160         170
ACGGGTAAGC CTGCCAACCA TGTTATGGCA GATTAAGCGT CTTTTTGAAG GGCACCGCAT

         190         200         210         220    A    230
CTTTCGCGTT GCCGTAAATG TATCCGTTTA TAAGGACAGC CCGAATGACG GTCTGCGCAA

         250         260         270         280         290
AAAAACACGT TCATCTCACT CGCGATGCTG CGGAGCAGTT ACTGGCTGAT ATTGATCGAC

         310         320         330         340         350
GCCTTGATCA GTTATTGCCC GTGGAGGGAG AACGGGATGT TGTGGGTGCC GCGATGCGTG

         370         380         390         400         410
AAGGTGCGCT GGCACCGGGA AAACGTATTC GCCCCATGTT GCTGTTGCTG ACCGCCCGCG

         430         440         450         460         470
ATCTGGGTTG CGCTGTCAGC CATGACGGAT TACTGGATTT GGCCTGTGCG GTGGAAATGG

         490         500         510         520         530
TCCACGCGGC TTCGCTGATC CTTGACGATA TGCCCTGCAT GGACGATGCG AAGCTGCGGC

         550         560         570         580         590
GCGGACGCCC TACCATTCAT TCTCATTACG GAGAGCATGT GGCAATACTG GCGGCGGTTG

         610         620         630         640         650
CCTTGCTGAG TAAAGCCTTT GGCGTAATTG CCGATGCAGA TGGCCTCACG CCGCTGGCAA

         670         680         690         700         710
AAAATCGGGC GGTTTCTGAA CTGTCAAACG CCATCGGCAT GCAAGGATTG GTTCAGGGTC

         730         740         750         760         770
AGTTCAAGGA TCTGTCTGAA GGGGATAAGC CGCGCAGCGC TGAAGCTATT TTGATGACGA

         790         800         810         820         830
ATCACTTTAA AACCAGCACG CTGTTTTGTG CCTCCATGCA GATGGCCTCG ATTGTTGCGA

         850         860         870         880         890
ATGCCTCCAG CGAAGCGCGT GATTGCCTGC ATCGTTTTTC ACTTGATCTT GGTCAGGCAT

         910         920         930         940         950
TTCAACTGCT GGACGATTTG ACCGATGGCA TGACCGACAC CGGTAAGGAT AGCAATCAGG

         970         980         990        1000        1010
ACGCCGGTAA ATCGACGCTG GTCAATCTGT TAGGCCCGAG GGCGGTTGAA GAACGTCTGA

        1030        1040        1050        1060        1070
GACAACATCT TCAGCTTGCC AGTGAGCATC TCTCTGCGGC CTGCCAACAC GGGCACGCCA

        1090        1100        1110        1120    B   1130
CTCAACATTT TATTCAGGCC TGGTTTGACA AAAAACTCGC TGCCGTCAGT TAAGGATGCT
```

FIG. 7 (a)

C

```
      1150        1160        1170        1180        1190
GCATGAGCCA TTTCGCGGCG ATCGCACCGC CTTTTTACAG CCATGTTCGC GCATTACAGA

      1210        1220        1230        1240        1250
ATCTCGCTCA GGAACTGGTC GCGCGCGGTC ATCGGGTGAC CTTTATTCAG CAATACGATA

      1270        1280        1290        1300        1310
TTAAACACTT GATCGATAGC GAAACCATTG GATTTCATTC CGTCGGGACA GACAGCCATC

      1330        1340        1350        1360        1370
CCCCCGGCGC GTTAACGCGC GTGCTACACC TGGCGGCTCA TCCTCTGGGG CCGTCAATGC

      1390        1400        1410        1420        1430
TGAAGCTCAT CAATGAAATG GCGCGCACCA CCGATATGCT GTGCCGCGAA CTCCCCCAGG

      1450        1460        1470        1480        1490
CATTTAACGA TCTGGCCGTC GATGGCGTCA TTGTTGATCA AATGGAACCG GCAGGCGCGC

      1510        1520        1530        1540        1550
TCGTTGCTGA AGCACTGGGA CTGCCGTTTA TCTCTGTCGC CTGCGCGCTG CCTCTCAATC

      1570        1580        1590        1600        1610
GTGAACCGGA TATGCCCCTG GCGGTTATGC CTTTCGAATA CGGGACCAGC GACGCGGCTC

      1630        1640        1650        1660        1670
GCGAACGTTA TGCCGCCAGT GAAAAAATTT ATGACTGGCT AATGCGTCGT CATGACCGTG

      1690        1700        1710        1720        1730
TCATTGCCGA ACACAGCCAC AGAATGGGCT TAGCCCCCCG GCAAAAGCTT CACCAGTGTT

      1750        1760        1770        1780        1790
TTTCGCCACT GGCGCAAATC AGCCAGCTTG TTCCTGAACT GGATTTTCCC CGCAAAGCGT

      1810        1820        1830        1840        1850
TACCGGCTTG TTTTCATGCC GTCGGGCCTC TGCGCGAAAC GCACGCACCG TCAACGTCTT

      1870        1880        1890        1900        1910
CATCCCGTTA TTTTACATCC TCAGAAAAAC CCCGGATTTT CGCCTCGCTG GGCACGCTTC

      1930        1940        1950        1960        1970
AGGGACACCG TTATGGGCTG TTTAAAACGA TAGTGAAAGC CTGTGAAGAA ATTGACGGTC

      1990        2000        2010        2020        2030
AGCTCCTGTT AGCCCACTGT GGTCGTCTTA CGGACTCTCA GTGTGAAGAG CTGGCGCGAA

      2050        2060        2070        2080        2090
GCCGTCATAC ACAGGTGGTG GATTTTGCCG ATCAGTCAGC CGCGCTGTCT CAGGCGCAGC

      2110        2120        2130        2140        2150
TGGCGATCAC CCACGGCGGC ATGAATACGG TACTGGACGC GATTAATTAC CGGACGCCCC

      2170        2180        2190        2200        2210
TTTTAGCGCT TCCGCTGGCC TTTGATCAGC CCGGCGTCGC GTCACGCATC GTTTATCACG

      2230        2240        2250        2260        2270
GCATCGGCAA GCGTGCTTCC CGCTTTACCA CCAGCCATGC TTTGGCTCGT CAGATGCGTT
```

FIG. 7 (b)

```
      2290        2300        2310        2320        2330
CATTGCTGAC CAACGTCGAC TTTCAGCAGC GCATGGCGAA AATCCAGACA GCCCTTCGTT

      2350        2360        2370        2380        2390
TGGCAGGGGG CACCATGGCC GCTGCCGATA TCATTGAGCA GGTTATGTGC ACCGGTCAGC

      2410        2420   E    2430      D 2440        2450
CTGTCTTAAG TGGGAGCGGC TATGCAACCG CATTATGATC TGATTCTCGT GGGGGCTGGA

      2470        2480        2490        2500        2510
CTCGCGAATG GCCTTATCGC CCTGCGTCTT CAGCAGCAGC AACCTGATAT GCGTATTTTG

      2530        2540        2550        2560        2570
CTTATCGACG CCGCACCCCA GGCGGGCGGG AATCATACGT GGTCATTTCA CCACGATGAT

      2590        2600        2610        2620        2630
TTGACTGAGA GCCAACATCG TTGGATAGCT CCGCTGGTGG TTCATCACTG GCCCGACTAT

      2650        2660        2670        2680        2690
CAGGTACGCT TTCCCACACG CCGTCGTAAG CTGAACAGCG GCTACTTTTG TATTACTTCT

      2710        2720        2730        2740        2750
CAGCGTTTCG CTGAGGTTTT ACAGCGACAG TTTGGCCCGC ACTTGTGGAT GGATACCGCG

      2770        2780        2790        2800        2810
GTCGCAGAGG TTAATGCGGA ATCTGTTCGG TTGAAAAAGG GTCAGGTTAT CGGTGCCCGC

      2830        2840        2850        2860        2870
GCGGTGATTG ACGGGCGGGG TTATGCGGCA AATTCAGCAC TGAGCGTGGG CTTCCAGGCG

      2890        2900        2910        2920        2930
TTTATTGGCC AGGAATGGCG ATTGAGCCAC CCGCATGGTT TATCGTCTCC CATTATCATG

      2950        2960        2970        2980        2990
GATGCCACGG TCGATCAGCA AAATGGTTAT CGCTTCGTGT ACAGCCTGCC GCTCTCGCCG

      3010        3020        3030        3040        3050
ACCAGATTGT TAATTGAAGA CACGCACTAT ATTGATAATG CGACATTAGA TCCTGAATGC

      3070        3080        3090        3100        3110
GCGCGGCAAA ATATTTGCGA CTATGCCGCG CAACAGGGTT GGCAGCTTCA GACACTGCTG

      3130        3140        3150        3160        3170
CGAGAAGAAC AGGGCGCCTT ACCCATTACT CTGTCGGGCA ATGCCGACGC ATTCTGGCAG

      3190        3200        3210        3220        3230
CAGCGCCCCC TGGCCTGTAG TGGATTACGT GCCGGTCTGT TCCATCCTAC CACCGGCTAT

      3250        3260        3270        3280        3290
TCACTGCCGC TGGCGGTTGC CGTGGCCGAC CGCCTGAGTG CACTTGATGT CTTTACGTCG

      3310        3320        3330        3340        3350
GCCTCAATTC ACCATGCCAT TACGCATTTT GCCCGCGAGC GCTGGCAGCA GCAGGGCTTT

      3370        3380        3390        3400        3410
TTCCGCATGC TGAATCGCAT GCTGTTTTTA GCCGGACCCG CCGATTCACG CTGGCGGGTT
```

FIG. 7 (c)

```
      3430        3440        3450        3460        3470
ATGCAGCGTT TTTATGGTTT ACCTGAAGAT TTAATTGCCC GTTTTTATGC GGGAAAACTC

      3490        3500        3510        3520        3530
ACGCTGACCG ATCGGCTACG TATTCTGAGC GGCAAGCCGC CTGTTCCGGT ATTAGCAGCA

                                F                   G
      3550        3560        3570        3580        3590
TTGCAAGCCA TTATGACGAC TCATCGTTAA AGAGCGACTA CATGAAACCA ACTACGGTAA

      3610        3620        3630        3640        3650
TTGGTGCAGG CTTCGGTGGC CTGGCACTGG CAATTCGTCT ACAAGCTGCG GGGATCCCCG

      3670        3680        3690        3700        3710
TCTTACTGCT TGAACAACGT GATAAACCCG GCGGTCGGGC TTATGTCTAC GAGGATCAGG

      3730        3740        3750        3760        3770
GGTTTACCTT TGATGCAGGC CCGACGGTTA TCACCGATCC CAGTGCCATT GAAGAACTGT

      3790        3800        3810        3820        3830
TTGCACTGGC AGGAAAACAG TTAAAAGAGT ATGTCGAACT GCTGCCGGTT ACGCCGTTTT

      3850        3860        3870        3880        3890
ACCGCCTGTG TTGGGAGTCA GGGAAGGTCT TTAATTACGA TAACGATCAA ACCCGGCTCG

      3910        3920        3930        3940        3950
AAGCGCAGAT TCAGCAGTTT AATCCCCGCG ATGTCGAAGG TTATCGTCAG TTTCTGGACT

      3970        3980        3990        4000        4010
ATTCACGCGC GGTGTTTAAA GAAGGCTATC TAAAGCTCGG TACTGTCCCT TTTTTATCGT

      4030        4040        4050        4060        4070
TCAGAGACAT GCTTCGCGCC GCACCTCAAC TGGCGAAACT GCAGGCATGG AGAAGCGTTT

      4090        4100        4110        4120        4130
ACAGTAAGGT TGCCAGTTAC ATCGAAGATG AACATCTGCG CCAGGCGTTT TCTTTCCACT

      4150        4160        4170        4180        4190
CGCTGTTGGT GGGCGGCAAT CCCTTCGCCA CCTCATCCAT TTATACGTTG ATACACGCGC

      4210        4220        4230        4240        4250
TGGAGCGTGA GTGGGGCGTC TGGTTTCCGC GTGGCGGCAC CGGCGCATTA GTTCAGGGGA

      4270        4280        4290        4300        4310
TGATAAAGCT GTTTCAGGAT CTGGGTGGCG AAGTCGTGTT AAACGCCAGA GTCAGCCATA

      4330        4340        4350        4360        4370
TGGAAACGAC AGGAAACAAG ATTGAAGCCG TGCATTTAGA GGACGGTCGC AGGTTCCTGA

      4390        4400        4410        4420        4430
CGCAAGCCGT CGCGTCAAAT GCAGATGTGG TTCATACCTA TCGCGACCTG TTAAGCCAGC

      4450        4460        4470        4480        4490
ACCCTGCCGC GGTTAAGCAG TCCAACAAAC TGCAGACTAA GCGCATGAGT AACTCTCTGT

      4510        4520        4530        4540        4550
TTGTGCTCTA TTTTGGTTTG AATCACCATC ATGATCAGCT CGCGCATCAC ACGGTTTGTT
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```
      4570        4580        4590        4600        4610
TCGGCCCGCG TTACCGCGAG CTGATTGACG AAATTTTTAA TCATGATGGC CTCGCAGAGG

      4630        4640        4650        4660        4670
ACTTCTCACT TTATCTGCAC GCGCCCTGTG TCACGGATTC GTCACTGGCG CCTGAAGGTT

      4690        4700        4710        4720        4730
GCGGCAGTTA CTATGTGTTG GCGCCGGTGC CGCATTTAGG CACCGCGAAC CTCGACTGGA

      4750        4760        4770        4780        4790
CGGTTGAGGG GCCAAAACTA CGCGACCGTA TTTTTGCGTA CCTTGAGCAG CATTACATGC

      4810        4820        4830        4840        4850
CTGGCTTACG GAGTCAGCTG GTCACGCACC GGATGTTTAC GCCGTTTGAT TTTCGCGACC

      4870        4880        4890        4900        4910
AGCTTAATGC CTATCATGGC TCAGCCTTTT CTGTGGAGCC CGTTCTTACC CAGAGCGCCT

      4930        4940        4950        4960        4970
GGTTTCGGCC GCATAACCGC GATAAAACCA TTACTAATCT CTACCTGGTC GGCGCAGGCA

      4990        5000        5010        5020        5030
CGCATCCCGG CGCAGGCATT CCTGGCGTCA TCGGCTCGGC AAAAGCGACA GCAGGTTTGA

                  H
      5050        5060        5070        5080        5090
TGCTGGAGGA TCTGATTTGA ATAATCCGTC GTTACTCAAT CATGCGGTCG AAACGATGGC

      5110        5120        5130        5140        5150
AGTTGGCTCG AAAAGTTTTG CGACAGCCTC AAAGTTATTT GATGCAAAAA CCCGGCGCAG

      5170        5180        5190        5200        5210
CGTACTGATG CTCTACGCCT GGTGCCGCCA TTGTGACGAT GTTATTGACG ATCAGACGCT

      5230        5240        5250        5260        5270
GGGCTTTCAG GCCCGGCAGC CTGCCTTACA AACGCCCGAA CAACGTCTGA TGCAACTTGA

      5290        5300        5310        5320        5330
GATGAAAACG CGCCAGGCCT ATGCAGGATC GCAGATGCAC GAACCGGCGT TTGCGGCTTT

      5350        5360        5370        5380        5390
TCAGGAAGTG GCTATGGCTC ATGATATCGC CCCGGCTTAC GCGTTTGATC ATCTGGAAGG

      5410        5420        5430        5440        5450
CTTCGCCATG GATGTACGCG AAGCGCAATA CAGCCAACTG GATGATACGC TGCGCTATTG

      5470        5480        5490        5500        5510
CTATCACGTT GCAGGCGTTG TCGGCTTGAT GATGGCGCAA ATCATGGGCG TGCGGGATAA

      5530        5540        5550        5560        5570
CGCCACGCTG GACCGCGCCT GTGACCTTGG GCTGGCATTT CAGTTGACCA ATATTGCTCG

      5590        5600        5610        5620        5630
CGATATTGTG GACGATGCGC ATGCGGGCCG CTGTTATCTG CCGGCAAGCT GGCTGGAGCA

      5650        5660        5670        5680        5690
TGAAGGTCTG AACAAAGAGA ATTATGCGGC ACCTGAAAAC CGTCAGGCGC TGAGCCGTAT
```

FIG. 7 (e)

```
      5710        5720        5730        5740        5750
CGCCCGTCGT TTGGTGCAGG AAGCAGAACC TTACTATTTG TCTGCCACAG CCGGCCTGGC

      5770        5780        5790        5800        5810
AGGGTTGCCC CTGCGTTCCG CCTGGGCAAT CGCTACGGCG AAGCAGGTTT ACCGGAAAAT

      5830        5840        5850        5860        5870
AGGTGTCAAA GTTGAACAGG CCGGTCAGCA AGCCTGGGAT CAGCGGCAGT CAACGACCAC

                                              L
      5890        5900        5910        5920        5930
GCCCGAAAAA TTAACGCTGC TGCTGGCCGC CTCTGGTCAG GCCCTTACTT CCCGGATGCG

                                          J
      5950        5960        5970        5980        5990
GGCTCATCCT CCCCGCCCTG CGCATCTCTG GCAGCGCCCG CTCTAGCGCC ATGTCTTTCC

      6010        6020        6030        6040        6050
CGGAGCGTCG CCTGAAGTTT TGACAGGGGC GGCGCATAGA GGAAGCCAAA AGAAACACAA

      6070        6080        6090        6100        6110
CCTTCTTTGC CCCTGACGGC GTGATGCATA CGGTGCGCCA TATACAACCG TTTGAGGTAG

      6130        6140        6150        6160        6170
CCCTTGCGTG GAATATAGCG GAATGGCCAA CGTTGATGCA CCAGCCCGTC GTGCACCATA

      6190        6200        6210        6220        6230
AAATAGAGTA ATCCATACGC CGTCATACCT GCGCCAATCC ACTGGAGCGG CCACATTCCT

      6250        6260        6270        6280        6290
GTACTGCCCA GATAAATCAG CAGGATCGAT AATGCAGCAA AAACCACGGC ATAAAGATCG

      6310        6320        6330        6340        6350
TTAACTTCAA ACGCACCTTT ACGCGGTTCA TGATGTGAAA GATGCCATCC CCAACCCCAG

      6370        6380        6390        6400        6410
CCGTGCATGA TGTATTTGTG TGCCAGTGCA GCAATCACTT CCATGCCAAT CACGGTAACG

                                  K
      6430        6440        6450        6460        6470
AAAACGATCA GGGCATTCCA AATCCACAAC ATAATTTCTC CGGTAGAGAC GTCTGGCAGC

      6490        6500        6510        6520        6530
AGGCTTAAGG ATTCAATTTT AACAGAGATT AGCCGATCTG GCGGCGGGAA GGGAAAAAGG

      6550        6560        6570        6580        6590
CGCGCCAGAA AGGCGCGCCA GGGATCAGAA GTCGGCTTTC AGAACCACAC GGTAGTTGGC

      6610        6620        6630        6640        6650
TTTACCTGCA CGAACATGGT CCAGTGCATC GTTGATTTTC GACATCGGGA AGTACTCCAC

      6670        6680        6690        6700        6710
TGTCGGCGCA ATATCTGTAC GGCCAGCCAG CTTCAGCAGT GAACGCAGCT GCGCAGGTGA

      6730        6740        6750        6760        6770
ACCGGTTGAA GAACCCGTCA CGGCGCGGTC GCCTAAAATC AGGCTGAAAG CCGGGCACGT

      6790        6800        6810        6820        6830
CAAACGGCTT CAGTACGGCA CCCACGGTAT GGAACTTACC GCGAGGCGCC AGGGCCGCAA
```

FIG. 7 (f)

```
      6850        6860        6870        6880        6890
AGTAGGGTTG CCAGTCGAGA TCGACGGCGA CCGTGCTGAT AATCAGGTCA AACTGGCCCG

      6910     6918
CCAGGCTTTT TAAAGCTT
```

FIG. 7 (g)

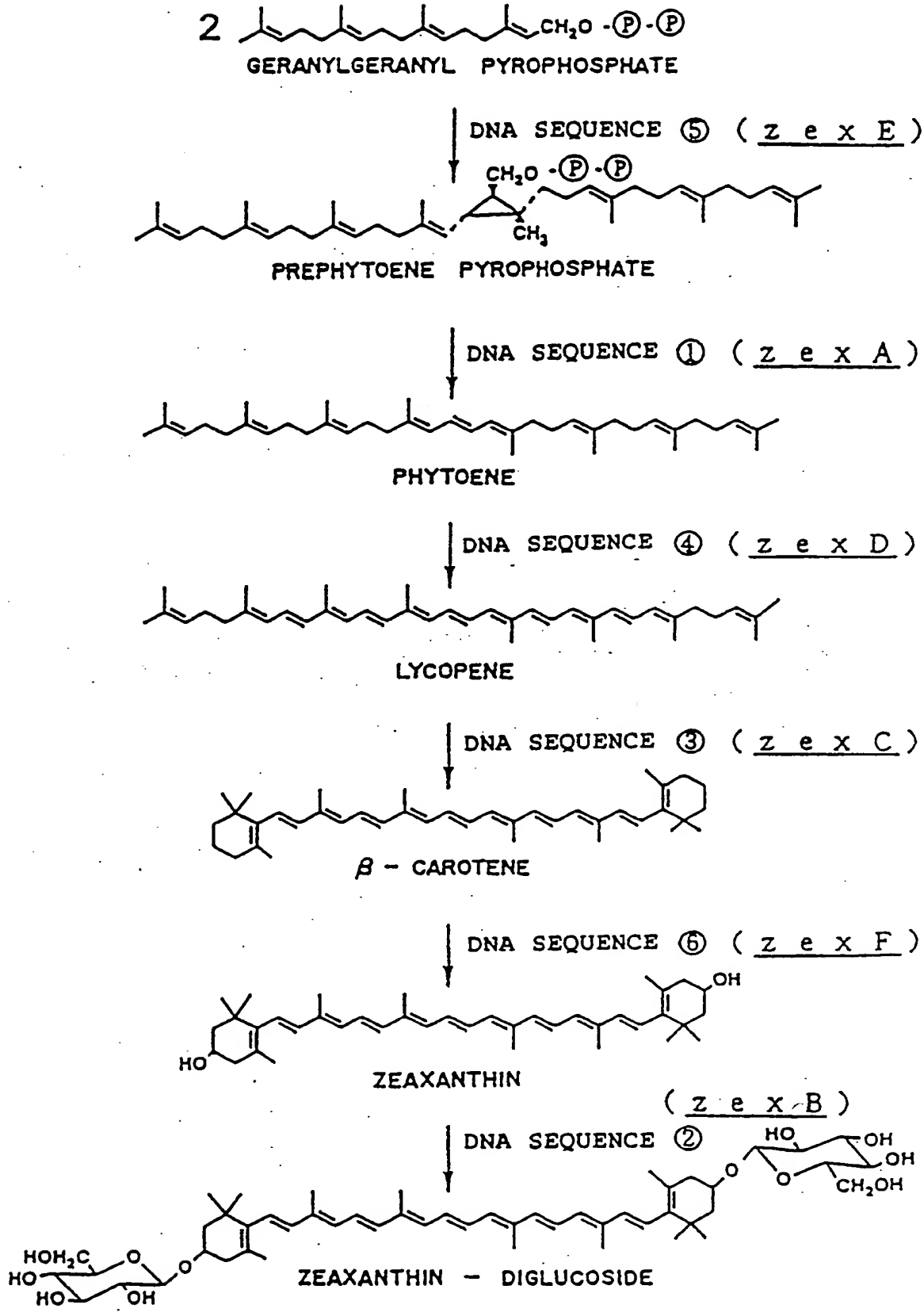
2
CH2O -Ⓟ-Ⓟ
GERANYLGERANYL PYROPHOSPHATE
DNA SEQUENCE ⑤ (z e x E)
CH2O -Ⓟ-Ⓟ
CH3
PREPHYTOENE PYROPHOSPHATE
DNA SEQUENCE ① (z e x A)
PHYTOENE
DNA SEQUENCE ④ (z e x D)
LYCOPENE
DNA SEQUENCE ③ (z e x C)
β - CAROTENE
DNA SEQUENCE ⑥ (z e x F)
OH
HO
ZEAXANTHIN
(z e x B)
DNA SEQUENCE ②
HO
OH
OH
O
CH2OH
HOH2C
O
HO
HO
OH
ZEAXANTHIN - DIGLUCOSIDE

FIG. 8

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Application Number

EP 90 10 7493

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	JOURNAL OF BACTERIOLOGY, vol. 168, no. 2, November 1986, pages 607-612, American Society for Microbiology; K.L. PERRY et al.: "Cloning and regulation of Erwinia herbicola pigment genes" ---		C 12 N 15/52 C 12 N 9/00 C 12 P 23/00 C 12 P 9/00 C 12 P 5/00
A	J. GEN. MICROBIOL., vol. 130, 1984, pages 1623-1631, SGM; G. THIRY: "Plasmids of the epiphytic bacterium Erwinia uredovora" ---		
A	CHEMICAL ABSTRACTS, vol. 109, no. 17, 24th October 1988, page 172, abstract no. 143512y, Columbus, Ohio, US; G. GIULIANO et al.: "A genetic-physical map of the Rhodobacter capsulatus carotenoid biosynthesis gene cluster", & MGG, MOL. GEN. GENET. 1988, 213(1), 78-83 ---		
A	CHEMICAL ABSTRACTS, vol. 110, no. 19, 8th May 1989, page 211, abstract no. 167285y, Columbus, Ohio, US; M.A NELSON et al.: "Molecular cloning of a Neurospora crassa carotenoid biosynthetic gene (albino-3) regulated by blue light and the products of the white collar genes", & MOL. CELL. BIOL. 1989, 9(3), 1271-6 --- -/-		TECHNICAL FIELDS SEARCHED (Int. Cl.5) C 12 N C 12 P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	10-07-1990	DESCAMPS J.A.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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L : document cited for other reasons

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EUROPEAN SEARCH REPORT

Application Number

EP 90 10 7493

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 5)
A	CHEMICAL ABSTRACTS, vol. 107, no. 7, 17th August 1987, page 222, abstract no. 53404u, Columbus, Ohio, US; J.M. PEMBERTON et al.: "Expression of Rhodopseudomonas sphaeroides carotenoid photopigment genes in phylogenetically related nonphotosynthetic bacteria", & CURR. MICROBIOL. 1987, 15(2), 67-71		

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	10-07-1990	DESCAMPS J.A.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
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P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
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